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The Pathophysiology of Diabetic Macular Oedema - a clinicopathological assessment.

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To Meena

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ABBREVIATIONS

Ang 1	Angiopoietin 1
Ang 2	Angiopoietin 2
BP	Blood Pressure
CSMO	Clinically Significant Macular Oedema
DM	Diabetes Mellitus
DMO	Diabetic Macular Oedema
DR	Diabetic Retinopathy
ET-1	Endothelin 1
ETDRS	Early Treatment Diabetic Retinopathy Study
s.Flt-1R	Soluble Flt-1 receptor
HGF	Hepatocyte Growth Factor
IL-1 Ra	Interleukin 1 receptor antagonist
IL-1 β	Interleukin 1 β
ILM	Internal Limiting Membrane
MVL	Medium Vision Loss
NO	Nitric Oxide
NPDR	Non-proliferative Diabetic Retinopathy
PDR	Proliferative Diabetic Retinopathy
PEDF	Pigment Epithelium Derived Factor
PPV	Pars plana vitrectomy
RPE	Retinal Pigment Epithelium
TGF β	Transforming Growth Factor β
UKPDS	UK Prospective Diabetic Study
VA	Visual Acuity
VEGF	Vascular Endothelial Growth Factor
ZO	Zonula occludens

ABSTRACT

Diabetic macular oedema (DMO) is a devastating vision-threatening complication of diabetes mellitus. The broad aim of the thesis is to investigate the hypothesis that vitreomacular traction and growth factors are important contributors to the development of DMO.

The contribution of vitreomacular traction was evaluated on the macular structural indices (foveal thickness and macular volume by Optical Coherence Tomography) and on functional effects (best corrected visual acuity) after 3-port pars plana vitrectomy (PPV) as part of clinical pilot studies with and without internal limiting membrane peels studies. The evaluation of growth factors in the vitreous, which was obtained at pars plana vitrectomy was performed using ELISA methods,. These vitreous samples from macular oedema patients (clinically graded as nonproliferative diabetic retinopathy NPDR) were compared to patients with full thickness macular holes (FTMH) and proliferative diabetic retinopathy (PDR). Lastly, using an immortalised rat retinal endothelial cell line, the effect of VEGF was explored to determine the molecular change at the junctional level.

In the non-randomised pilot series of PPV, there was a significant improvement in the structural and functional indices at 12 months compared to baseline ($p=0.037$, $p=0.01$ respectively). However, in the pilot randomised and nonrandomised study including ILM peel, surgery provided little visual improvement despite structural benefit. These clinical studies of pars plana vitrectomy do suggest a role of vitreomacular traction in the development of macular oedema. Vitreal VEGF-A and HGF (angiogenic) concentrations were increased with a corresponding decrease in the angiostatic agents (soluble Flt-1 R antibody and PEDF). There were also changes in the angiopoietin 1 and 2 concentrations with significantly lower concentrations of angiopoietin 1 in macular oedema, suggesting a lower anti-permeability protective effect of angiopoietin 1. Haemodynamic (endothelin-1) and inflammatory (Il-1 β) markers in the patients with macular oedema also demonstrated changes compared to control patients especially in endothelin-1 where there was a significant decrease in its concentration in the diabetic macular oedema.

The effect of VEGF on cultured immortalised retinal endothelial cells (with primary rat endothelial culture acting as a comparison) did demonstrate that high concentrations of VEGF (100 ng/ml) could disrupt the organisation of tight junctions.

These results demonstrate that the development of diabetic macular oedema is multifactorial with a range of physical (vitreomacular) and biochemical (cytokine) forces acting on and within the retina to produce leakage of fluid into the macula.

CHAPTER 1

INTRODUCTION

CHAPTER 1.1 DIABETES MELLITUS

1.1.1 Diabetes mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycaemia secondary to relative or absolute deficiency of insulin secretion, resistance to insulin action, or both (Gerich 2003). Although several pathogenic mechanisms maybe involved in the development of DM, the vast majority fall into two distinct groups: Type 1 diabetes, previously known as 'insulin dependent' or 'juvenile onset' is characterized by destruction of the insulin secretory pancreatic β-cells of the islet of Langerhans caused by an autoimmune process usually leading to insulin deficiency and the need to replace insulin (Atkinson and Eisenbarth 2001). Type II diabetes ('non-insulin dependent' or 'adult onset') is characterized by insulin resistance in peripheral tissues and an insulin secretory defect of β-cells (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1997). 70-90 % of diabetic patients have type II diabetes (Bennett *et al.* 1995). Insulin resistance develops as a consequence of obesity, sedentary lifestyle and ageing with resulting hyperglycaemia and diabetes, blood pressure elevation and dyslipidaemia. Indeed, collectively these abnormalities which often occur together have been designated the 'dysmetabolic syndrome' (Skyler 2004). Consequently in both types a general disruption of carbohydrate, protein and fat metabolism begins which ultimately produces tragic ravages of long-term damage, dysfunction and failure to eyes, kidneys, nerves, heart and major blood vessels.

The World Health Organization and the International Diabetes Federation have estimated that there is a global population of 194 million people with diabetes, representing a global prevalence exceeding 3 % of the world population (Federation 2003; Organization 2003). It's also been estimated there will be an increase in the global population with diabetes to 333 million, equivalent to 6.3 %, by 2025, with the most significant increase in the developing countries, probably as a result of population growth, obesity, ageing and sedentary lifestyles (King *et al.* 1998).
The corresponding health cost of diabetes in the UK, not only in relation to the management of the disease but also its complications, was £5.2 billion representing

9% of the annual National Health Service (NHS) budget in 2000.

1.1.2 Diabetic Retinopathy.

Diabetic retinopathy (DR) is a progressive disease predominantly affecting the retinal microvasculature producing diabetic retinopathy (DR). DR can be broadly divided into two clinical stages: non-proliferative (NPDR) and proliferative DR (PDR). In NPDR, the disease process generally involves changes in the permeability of the retinal blood vessels (resulting in the collection of solutes and fluid in the retina and producing macular oedema with secondary loss of visual acuity) and capillary closure leading to the formation of microaneurysms. Consequently in NPDR venous dilatation, microaneurysms, retinal haemorrhages, retinal oedema, and hard exudates are seen clinically. PDR develops from capillary occlusion producing retinal ischaemia, which promotes the development of neovascularization, a process by which fragile new blood vessels grow from existing retinal vessels. Given the fragility of these vessels, they haemorrhage easily leading to vitreous haemorrhage and tractional retinal detachment, with both complications resulting in loss of vision (Cogan *et al.* 1961) (Engerman 1989).

International Clinical Diabetic Retinopathy Disease Severity and Diabetic Macular Oedema Severity Scales have been proposed which were derived from more research orientated scales (The Early Treatment Diabetic Retinopathy Study (Early Treatment Diabetic Retinopathy Study Research Group 1991) and the Wisconsin Epidemiologic Study of Diabetic Retinopathy (Klein *et al.* 2001) scales).

Table 1.1: International clinical diabetic retinopathy severity scale.

Proposed Disease Severity Level	Dilated Ophthalmoscopic findings
No apparent retinopathy	No abnormalities
Mild nonproliferative DR	Microaneurysms
Moderate nonproliferative DR	More than just microaneurysms but less than severe NPDR, retinal haemorrhages, cotton wool spots
Severe nonproliferative DR	No signs of PDR with any of the following: 1. More than 20 intraretinal haemorrhages in each of four quadrants 2. Definite venous beading in two or more quadrants 3. Prominent intraretinal microvascular anomalies in one or more quadrants
Proliferative DR	One or more of the following 1. Neovascularization – at the disc or elsewhere 2. Vitreous or preretinal haemorrhage

The prevalence of any form of DR in Type I diabetes at diagnosis lies between 0-3 % (Klein *et al.* 1997). For Type II the prevalence at diagnosis is higher between 6-30% (Aiello *et al.* 1998). For both Type I and Type II the prevalence of DR strongly correlates with duration of diabetes: for Type I duration of < 2 years duration there is a prevalence of 2 % and this rises to a prevalence of 98 % for duration of > 15 years; for Type II the equivalent figures are 20-23 % prevalence for < 2 years and 58-85 %

prevalence for a duration of > 15 years (Klein 1987).

Some patients develop visual loss secondary to macular oedema. Clinically significant macular oedema (CSMO) occurs if retinal thickening occurs within 500 μm of the fovea, if there are hard exudates at or within 500 μm of the fovea with thickening of the adjacent retina or if there is a zone of retinal thickening one disc area or larger any part of which is within one disc diameter of the fovea. The majority of visual loss occurs when macular oedema reaches the fovea and so CSMO represents the threshold level at which laser photocoagulation is performed (see later) (Early Treatment Diabetic Retinopathy Study Research Group 1985).

The prevalence of macular oedema (MO) is also related to the duration of disease. In the first 5 years following diagnosis of Type I diabetes, macular oedema did not develop in the patients compares to 29% developing it after 20 years (Klein 1987). For Type II diabetes, 3 % develop MO in the first 5 years of the disease process but after 20 years of duration, 28 % develop MO (Klein 1987). The natural history of macular oedema in Type 1 and Type 2 patients is characterised by progressive visual loss with over half of affected patients losing 2 or more lines of visual acuity (VA) after 2 years of follow up with a greater effect on the older onset patient: 55% of this type of patient with macular oedema has visual acuity worse than 6/9, compared with only 20% of the younger onset patient (Klein *et al.* 1984).

Table 1.2: International clinical diabetic macular oedema disease severity scale

Proposed Disease Severity Level	Dilated Ophthalmoscopic findings
Mild:	some retinal thickening or hard exudates in the posterior pole but distant from fovea
Moderate:	retinal thickening or exudates approaching the fovea but not involving it.
Severe:	retinal thickening or exudates involving the fovea.

1.1.3 Pathophysiology

Many studies have demonstrated that chronic hyperglycaemia as well as hyperlipidaemia and hypertension contribute to the pathogenesis of DR (Klein *et al.* 1988), (Vitale *et al.* 1995 26), (Chew *et al.* 1996), (Klein *et al.* 1998). The exact mechanisms by which elevated glucose initiates the vascular disruption in retinopathy remain poorly defined and several pathways have been implicated.

The vascular disruptions are characterized by abnormal vascular flow, disruption in permeability and or closure or non-perfusion of capillaries. The corresponding structural changes involve damage to the microvasculature with both endothelial and pericyte cell damage, and thickening of the capillary basement membrane and increased deposition of extracellular matrix components. Endothelial cells provide one element of the blood retinal barrier and so damage to them leads to increased vascular permeability. Pericytes control retinal capillary perfusion and therefore damage to them produces altered retinal haemodynamics and impaired autoregulation as well as correlating with microaneurysm formation (Cogan *et al.* 1961). A change to the basement membrane also alters retinal haemodynamics.

The biochemical pathways which lead from a state of hyperglycaemia to vascular disruption are multiple and all may be considered a component in the pathogenesis of vascular damage and as a consequence in the development of macular oedema. These pathways include metabolic alterations, haemodynamic changes, role of leukostasis, mechanical or tractional forces from the vitreous body and the role of angiogenic growth factors.

1.1.3.1 Metabolic Alterations

Hyperglycaemia induces changes in the normal metabolic pathways with the development of new metabolic processes that include activation of the sorbitol-polyol pathway, the production of oxygen and peroxynitrite free radicals, the initiation of non-enzymatic glycation and the activation of the diacylglycerol-protein kinase C pathway.

1.1.3.1.1 Sorbitol-Polyol Pathway

Hyperglycaemia raises intracellular glucose levels in insulin-independent tissues such as nerve, glomerulus, lens and retina. In these tissues the enzyme aldose reductase catalyses the glucose to sugar alcohol or sorbitol. Glucose itself is a poor substrate for aldose reductase (glucose has a high binding constant for this enzyme). Therefore aldose reductase is not operative in this pathway until the glucose concentrations, as in hyperglycaemia, are very high by which time the usual pathways of glucose metabolism through the enzyme hexokinase pathway of glycolysis, which ultimately leads to the formation of lactic acid, are saturated. This increased flux through the sorbitol pathway in a state of hyperglycaemia can account for as much as one-third of the total glucose turnover Sorbitol itself is catalysed to fructose using sorbitol dehydrogenase and NAD^+ as cofactor **(see Fig 1.1 a).**

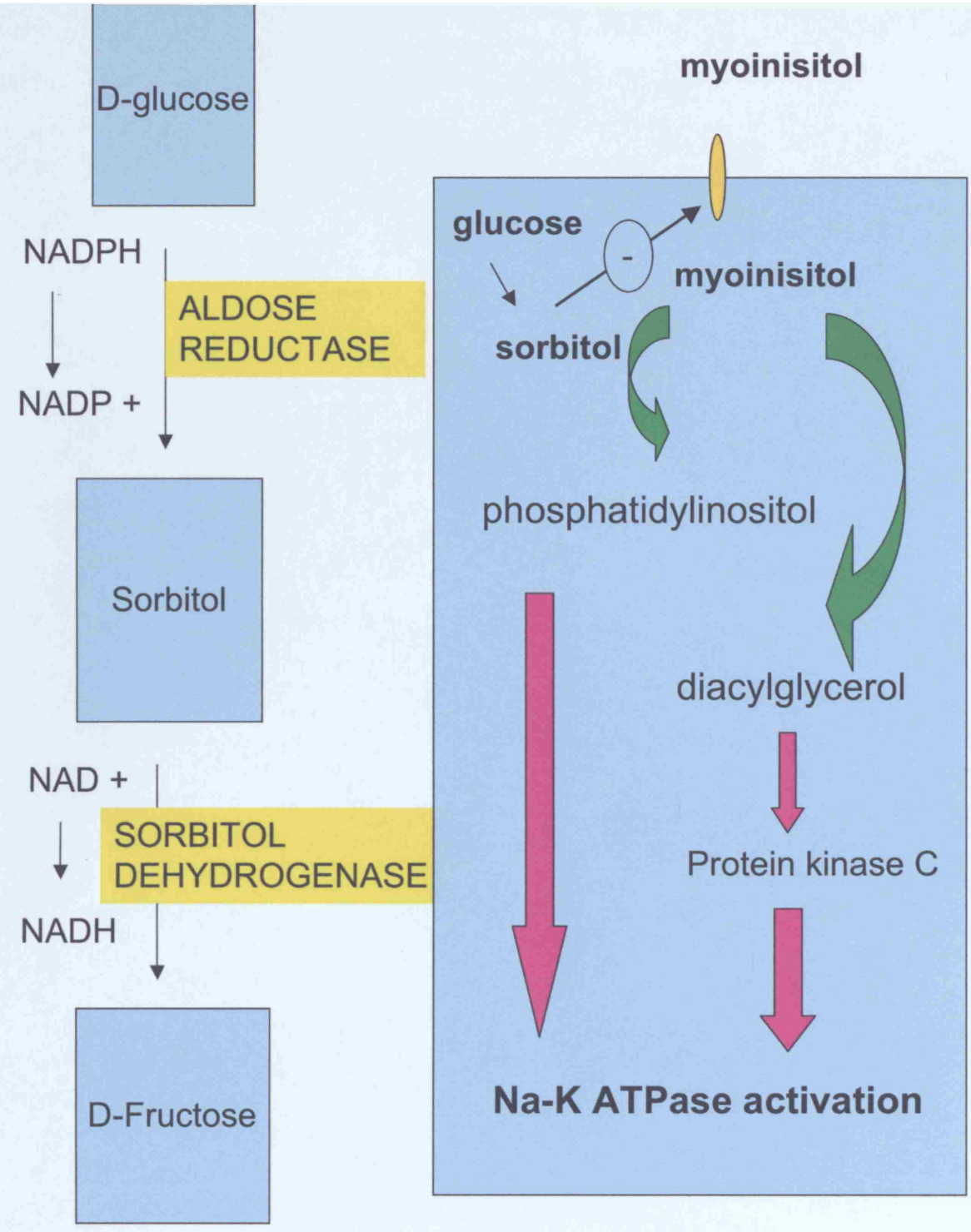


Fig 1.1 a

Fig 1.1 b

Figure 1.1 Abnormal Glucose Metabolic Pathways in Hyperglycaemia

a) Sorbitol pathway activity during the state of hyperglycaemia

b) The Myoinisitol pathway and its inhibition by sorbitol

Sorbitol does not easily cross cell membranes and accumulates intracellularly. It can either produce cellular damage through its osmotic effects (e.g. in the lens), by increasing the NADH / NAD^+ ('pseudohypoxia') and by depleting intracellular myoinositol (Williams and Pickup 1999). Also as the latter reaction can occur very slowly then sorbitol levels can increase to toxic concentrations.

Myoinositol is structurally related to glucose and is a precursor of phosphatidylinositol, which directly or through diacylglycerol activates Na^+- K^+- ATPase. Glucose and sorbitol may compete with myoinositol, blocking its uptake into cells, and consequently decreasing Na^+- K^+- ATPase activity. The depletion of myoinisitol has been implicated in the physiological impairment of retinal pigment epithelial cells (RPE) and pericytes by hyperglycaemia (Del Monte *et al.* 1991) (Li *et al.* 1991) **(see Fig 1.1 b)**

Pseudohypoxia can in itself lead to cellular imbalances in lipid metabolism, the formation of free radicals, increased Diacylglycerol (DAG) synthesis, decreased nitric oxide synthesis and protein kinase C activation by DAG which at a tissue level can lead to increased blood flow, hyperpermeability and abnormal growth factor expression (Archer 1999) **(see Fig 1. 2)**.

1.1.3.1.2 Oxidative damage **(see Fig 1. 2)**

Chronic hyperglycaemia can also produce accelerated oxidative stress in cells leading to the formation of excessive toxic end products of oxidation. These include peroxides, superoxide and oxygen free radicals (Pruthi *et al.* 2001). Peroxynitrite and hydroxyl radicals are also formed as a consequence of lipoxygenase enzyme activation by the state of hyperglycaemia. These cascades of reactive radical formation are coupled with a decrease in the antioxidant defence mechanisms. There is a decrease in glutathione levels, superoxide dismutase and catalase activities in animal models of diabetes and galactosaemia (Chakrabarti *et al.* 2000). This superoxide production emanates from the mitochondrial electron-transport chain (Brownlee 2000).

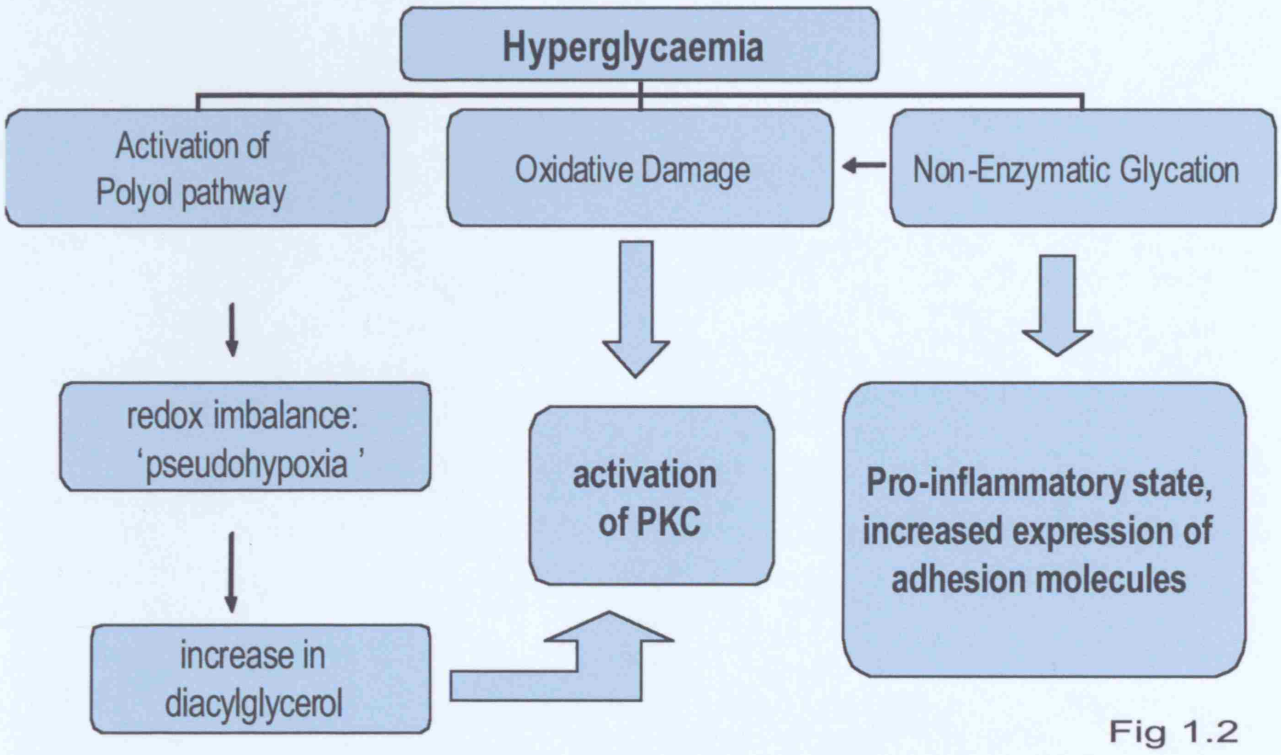


Figure 1.2 Hyperglycaemia Induced Biochemical Changes

Summary of three biochemical pathways activated by hyperglycaemia

Principally, oxidative stress contributes to diabetic vascular dysfunction. The mechanism by which this is achieved is complex but involves activation of nitric oxide synthase and production of nitrogen reactive species, particularly peroxynitrite. A working model describes the overproduction of superoxide induced by hyperglycaemia. This leads to an increase in intracellular calcium and activation of

nitric oxide synthase (NOS 3) to form nitric oxide, but also uncoupling of NOS 3 (Hammes 2003), which leads to further superoxide production.
Superoxide combines with nitric oxide to form peroxynitrite, which deactivates nitric oxide (Graier *et al.* 1996). Peroxynitrite induces extensive damage at the cellular level to protein systems, loss of cellular antioxidant defences, lipid peroxidation and induction of DNA damage leading to apoptosis (Salgo *et al.* 1995a; Salgo *et al.* 1995b). Other pathways the free radicals also activate are those mediated by aldose reductase, and PKC. These promote non-enzymatic glycation of proteins.

1.1.3.1.3 Non-Enzymatic Glycation (**see Fig 1. 2**)

Glycation of proteins as a consequence of hyperglycaemia is analogous to glycated haemoglobin (HbA1c). Glucose in cells and in extracellular spaces attach non-enzymatically to amino groups on proteins, which then undergo a biochemical rearrangement to a more stable glycation product. Irreversible glycation of proteins is called the Maillard reaction, whilst non-stable glycation products are also known as 'Amadori products', which are reversible until they become more stable, and permanent by forming cross-linked products called 'advanced glycation end-products' (AGE).

Excessive formation of AGE has been proposed as another biochemical link between diabetes and the development of microvascular complications. AGE can react with cell surface receptor proteins (AGE-specific receptors) and induce a cocktail of biochemical changes. They can interfere with normal vascular function, and promote a pro-coagulant state, which includes altering blood viscosity, and tissue oxygenation. Tissue oxygenation is disturbed because of its effects on haemoglobin. An increase in HbA1c is associated with a decrease in 2,3-diphoshoglyceric acid within the red blood cell. Both these products increase the affinity of haemoglobin for oxygen and so decrease oxygen release to the tissue resulting in tissue hypoxia especially in the venous side of the circulation. The propensity of AGE formation to occur in structural proteins such as collagen also leads to the interference with extracellular matrix function and an increase its thickness. AGE can also perpetuate a pro-inflammatory state with the expression of adhesion molecules on endothelial cells and leucocytes. This increased expression of adhesion molecules together with decreased

deformability of the leucocytes in diabetes can result in leukocyte adhesion, leukostasis and subsequent focal thrombosis and capillary closure (Archer 1999) (Brownlee 2000). Within vascular endothelial cells, AGE can impair both the activity of nitric oxide (NO) and expression of endothelial nitric oxide synthase (eNOS), which may contribute to the failure of autoregulatory control in diabetic retinal vasculature. This can lead to vasoconstriction of the retinal vasculature (Brownlee 2000).

1.1.3.1.4 Protein Kinase C activation *(PKC)* **(see Fig 1.2 and 1.3)**.

Studies by Xia et al have shown that within a few months of the onset of diabetes there is substantial elevation of diacylglycerol (DAG) within the cells of the retina and the aorta (Xia *et al.* 1994). Diabetes-induced DAG may derive from hydrolysis of phosphatidylinositides, which themselves are derived from myoinisitol, or *de novo* synthesis of phosphatidic acid (Koya and King 1998). PKC activity can also be increased after exposure of vascular endothelial cells to oxidative stress (Taher *et al.* 1993).The δ and β II isoforms of PKC are particularly activated in vascular tissues in response to hyperglycaemia (Koya and King 1998). PKCβ II has been shown to have an important role in regulating endothelial cell permeability, basement membrane synthesis and vessel contractility by modulating the balance between vasodilatory and vasoconstrictive agents and coupled with changes in coagulation can affect retinal blood flow (Archer 1999).

1.1.3.2 Hydrostatic Forces / Haemodynamic Theory **(Fig 1.4)**

Accompanying the biochemical processes outlined above, and in some instances possibly preceding microangiopathy, changes in the hydrostatic forces and haemodynamic and rhelogical properties of blood flow and blood are seen.

Autoregulation refers to a homeostatic process that maintains constant pressure in the retinal capillaries across a range of blood pressure values. In diabetic retinopathy this process becomes impaired mainly because of the impaired endothelial metabolism induced by hyperglycaemia as outlined above. Hypoxia and systemic arterial

hypertension can also damage the autoregulatory process. As the autoregulatory processes breakdown, the arterioles begin to dilate (according to Poiseuille's law of pressure). This transmits a greater hydrostatic pressure to the capillary bed, which overpowers the counterbalancing force of retinal tissue osmotic pressure allowing the development of oedema proportional to the overall permeability of the vessel wall (according to Starling's law) (Gardner *et al.* 2002).

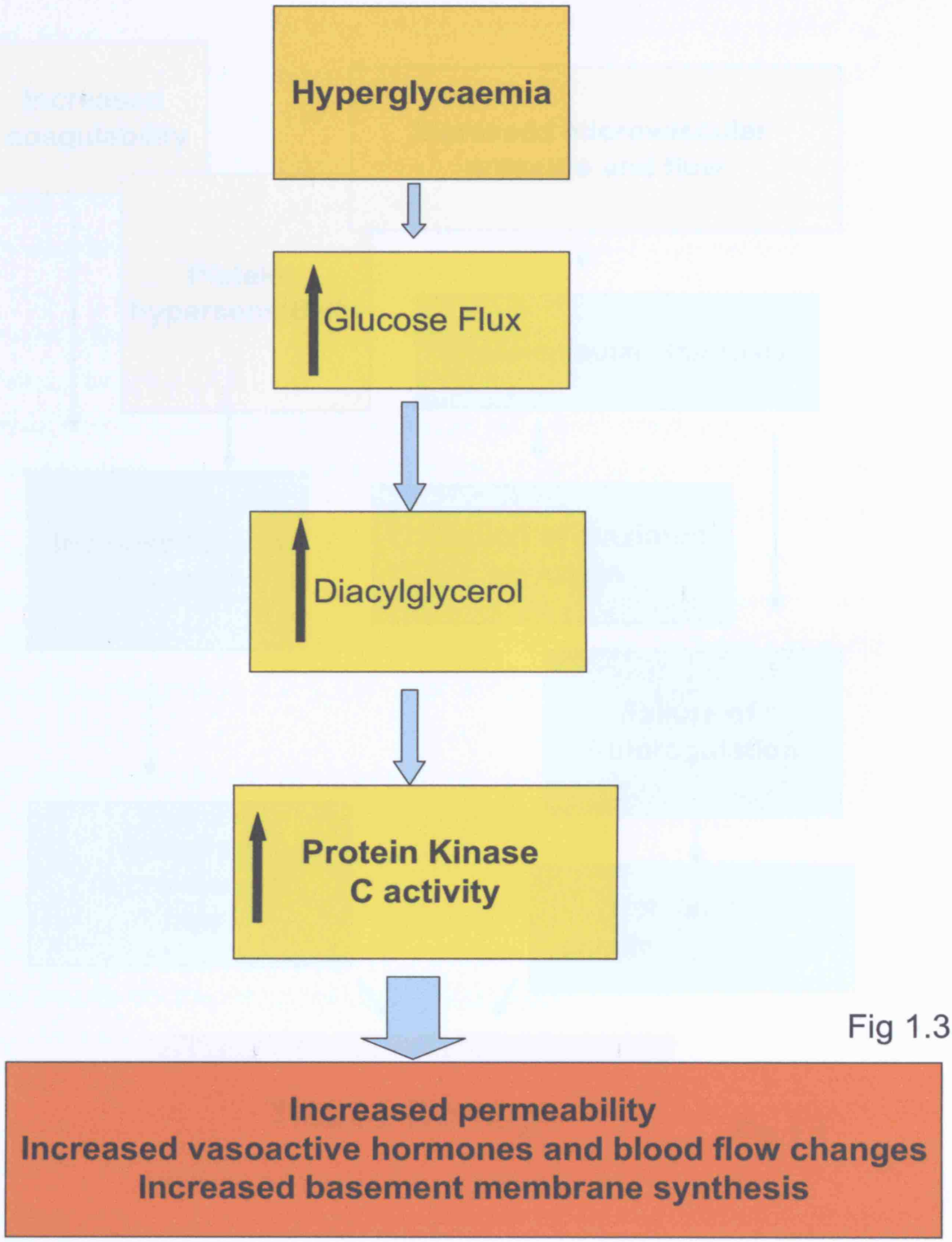


Fig 1.3

Figure 1.3 Hyperglycaemia induced PKC activity

Pathway of Protein Kinase C activity and subsequent changes induced by PKC.

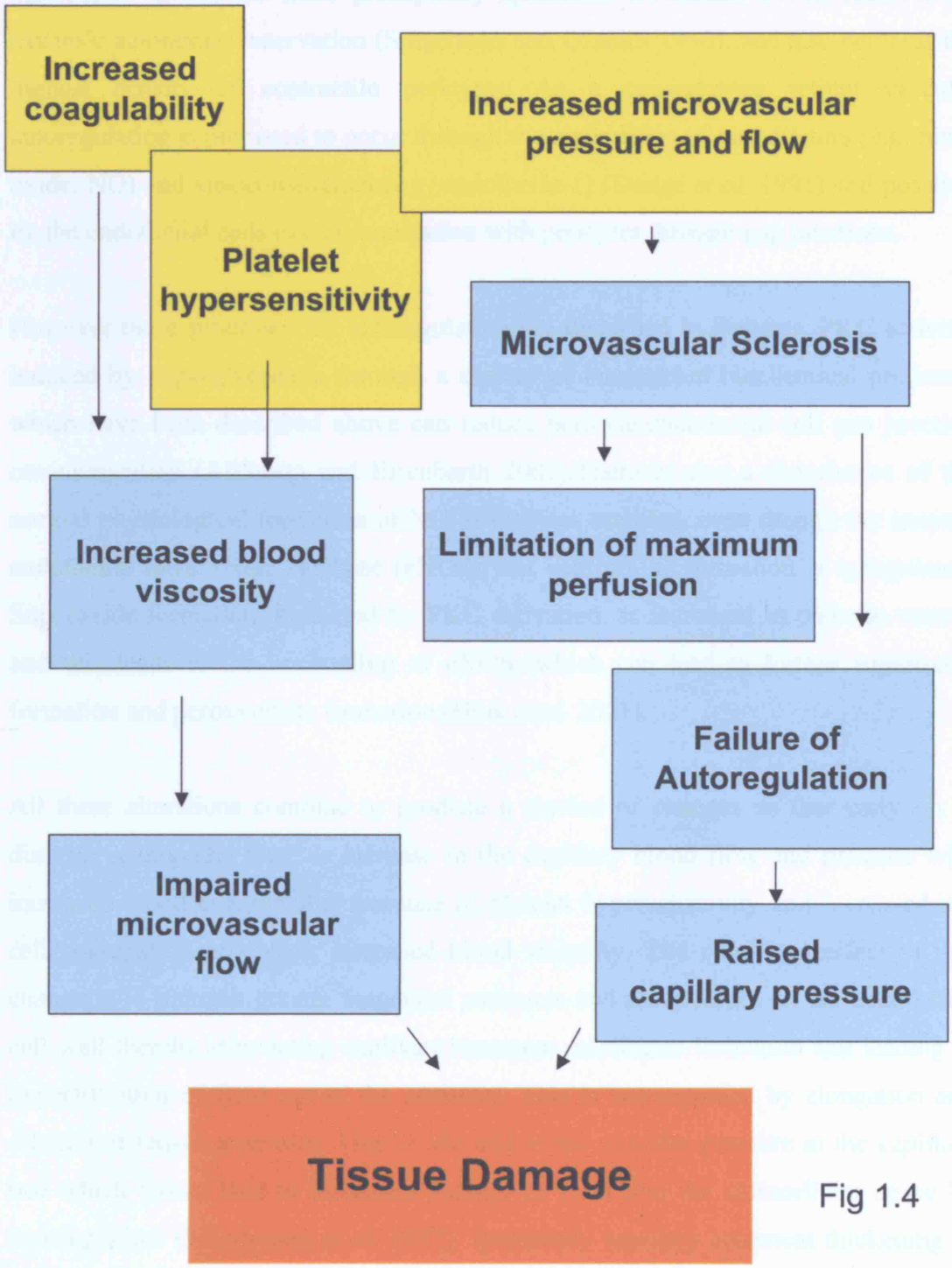


Fig 1.4

Figure 1.4 Haemodynamic and Rheological Changes in Diabetes Mellitus.
Changes in the haemodynamic and rheological parameters by diabetes and subsequent pathways leading to tissue damage.

Retinal blood flow autoregulation largely occurs at the retinal capillary level because the retinal vasculature lacks precapillary sphincters (Friedman 1970), receives no extrinsic autonomic innervation (Singelman and Ozanics 1990), and also contains the highest density of contractile pericytes. As a consequence retinal vascular autoregulation is proposed to occur through the elaboration of vasodilators (e.g. nitric oxide, NO) and vasoconstrictors (e.g. endothelin-1) (Dodge *et al.* 1991) and possibly by the endothelial cells in communication with pericytes through gap junctions.

However these processes for autoregulation are disturbed in diabetes. PKC activity, induced by hyperglycaemia through a system of interrelated biochemical processes which have been described above can reduce pericyte-endothelial cell gap junction communication (Atkinson and Eisenbarth 2001).There is also a disturbance of the normal physiological formation of NO in diabetes mellitus, even though the enzyme endothelial nitric oxide synthase (eNOS) that controls its formation is upregulated. Superoxide formation, mediated by PKC activation, is increased in diabetic vessels and this leads to the uncoupling of eNOS, which can lead to further superoxide formation and peroxynitrite formation (Hink *et al.* 2001).

All these alterations combine to produce a myriad of changes so that early on in diabetic retinopathy there is increase in the capillary blood flow and pressure with increased blood coagulability because of platelet hypersensitivity and increased red cell aggregation producing increased blood viscosity. The combined effect of this change is to transmit greater tangential pressures and shear forces on the endothelial cell wall thereby stimulating capillary basement membrane formation and leading to hyperfiltration of fluid out of the capillary. This is accompanied by elongation and dilation of retinal arterioles. This would add to the vascular pressure in the capillary bed which would lead to increased passage of fluid into the extracellular space by Starling's law (Kristinsson *et al.* 1997). Eventually capillary basement thickening (a hallmark of diabetic retinopathy) limits vasodilatation, further impeding autoregulation and with the rheological changes impairing microvascular flow and tissue damage **(see Fig 1.4)**.

The haemodynamic theory also comprises of changes in blood rheology which produces retinal leukostasis (McLeod *et al.* 1995), blood viscosity (McMillan 1983)

and erythrocyte deformability (Miller *et al.* 1980). The retinal leukostasis theory suggests that endothelial cell death and retinal vascular leakage are secondary to the adherence of activated leukocytes. A significant increase in the density of retinal granulocytes has been observed in human diabetics (McLeod *et al.* 1995). This adherence is mediated by an increase in the expression of intercellular adhesion molecule – 1 (ICAM-1) on the retinal endothelial cells (McLeod *et al.* 1995). Furthermore ICAM-1 expression is increased by the diabetic microenvironmental conditions of the retina (e.g. the hypoxia, hyperglycaemia (Taki *et al.* 1996), AGE (Vlassara *et al.* 1995) and shear stress (Nagel *et al.* 1994). The adherent granulocytes can produce focal capillary occlusion with subsequent focal retinal ischaemia. Platelet abnormalities may also cause similar focal capillary and ischaemic changes (McMillan 1983). Capillary closure can lead to hypoxic damage to the endothelium and elaboration of angiogenic growth factors e.g. vascular endothelial growth factor (VEGF). The presence of these activated leucocytes would lead to the release of inflammatory cytokines e.g. interleukin 1 beta (Il-1 β) and prostacyclin which may contribute to the endothelial damage and leukostasis (Miyamoto and Ogura 1999). The negating effect of interleukin 1 receptor antagonist (Il-1Ra) and other down-regulators of inflammation maybe compromised in the diabetic state, but no evidence exists for this.

Blood viscosity is also increased in diabetics due to glycosylation-induced changes in the red blood cells. The shear stress induced by the increased blood viscosity can also damage the endothelial cells and cause leakage form the retinal vessels. The retinal blood flow is also decreased not only from these changes but also because of the reduced ability of erythrocytes to change their shape induced by glycosylation of their cell membranes (Merimee 1990).

Furthermore hyperglycaemia induces pericyte and endothelial cell apoptosis (Mizutani *et al.* 1996).This consequence of diabetes is related to its ability to alter vascular cell kinetics. At a cellular level, hyperglycaemia is able to directly or indirectly influence cell proliferation, migration and angiogenesis on one hand or cell dysfunction and death on the other.

Ordinarily vascular cell replication occurs at a very low rate, just to replace wear and

tear damage to the vascular lining (Sharma *et al.* 1985). In severely diabetic animals, vascular endothelial cell turnover is vastly increased with pericytes showing a diminished replicative capacity (Sharma *et al.* 1985). This is seen clinically as an area of intraretinal microvascular anomalies and provides the basis for new vessel formation.

However, in contrast, Sharma et al also demonstrated increased endothelial cell death in the diabetic rat model when they studied the distribution of ^{3}H- thymidine labelled cells in trypsin digests (Sharma *et al.* 1985). This description of increased cell death in diabetes was confirmed from work in diabetic patients and experimental animals which showed that apoptosis is the chief mode of endothelial cell and pericyte death in diabetes by confirming an increase in terminal deoxynucleotidyl transferase-mediated d-UTP nick end labelling (TUNEL) in retinal trypsin digests of diabetic retinopathy (Mizutani *et al.* 1996) which may even precede histological evidence of retinopathy.

1.1.3.3 Summary of Metabolic and Haemodynamic Changes

In summary the above description of the various biochemical, haemodynamic and vascular cellular changes are interrelated. The expression of the sorbitol pathway would lead to increased oxidative free radical formation, diacylglycerol and protein kinase C activation which has been shown to regulate cell permeability (Nagpala *et al.* 1996) and vessel contractility. The free radicals can induce cellular damage, and promote glycation of proteins with AGE formation. AGE formation either stimulated directly by hyperglycaemia or indirectly (by free radicals) damages intracellular molecules (e.g. DNA) as well as extracellular functional and structural proteins (e.g. collagen). They also promote increased blood viscosity, the development of a pro-inflammatory state and impair nitric oxide activity, thereby damaging autoregulation of retinal blood flow. This impaired autoregulation is a function of reduced nitric oxide formation (either by AGE activity or free radical damage) and changes in vascular cell kinetics with increased apoptosis. This would alter retinal blood flow and by Starling's Law result in a change of flow of fluid into the extracellular space. The alterations in hydrostatic forces are coupled with changes also in endothelin

formation, which later in diabetes induces sufficient vessel constriction possibly to reduce blood flow. This in turn accompanies changes in white cell and red blood cell flow through the capillaries. The retinal capillaries themselves elaborate increased adherence proteins making them more 'sticky' (McLeod *et al.* 1995) and so promoting leukostasis and together with reduced red cell deformability and increased blood viscosity focal closure of capillaries can occur.

The above model may provide a sufficient basis for the pathogenesis of diabetic retinopathy and macular oedema, given the far-reaching changes they cause to the vascular endothelium, pericyte and blood flow, all of which are seen in diabetic retinopathy. However, these biochemical theories do not account for the retinal ischaemia and new vessel formation seen in severe non-proliferative and proliferative stages of diabetic retinopathy. Furthermore when clinical trials were conducted looking at the role of specific inhibitors of components in the biochemical theory, the results suggested that these components by themselves may not provide the complete answer to the pathogenesis of diabetic retinopathy (DR), but maybe part of a series of steps which leads to the development of a process which by itself is then able to promote the clinical retinal damage seen.

When specific aldose reductase inhibitors were used in clinical trials, they showed little therapeutic benefit. Even though decreases in microaneurysm count (Sorbinil Retinopathy Trial Research Group 1990) and fluorescein leakage (van Gerven *et al.* 1994) were seen in the patients, these effects did not alter the progression of DR (Tromp *et al.* 1991).

The use of aminoguanidine, an inhibitor of AGE formation, has shown promising results in animal models (including such effects as reduction in number of acellular capillaries and pericyte loss (Hammes *et al.* 1991) and also oxidative stress and protein kinase C activation (Kowluru *et al.* 2000) in rodent models of diabetes, whilst in a corresponding dog model it prevented the development of DR (Kern and Engerman 2001). However, in humans the use of these compounds for the prevention of DR was not as effective as described in the animal models (Freedman *et al.* 1999).

Ruboxistaurin (LY333531), a specific inhibitor of PKC- $\beta 1$ and – $\beta 2$ has been shown

to prevent and reverse microvascular complications in animal models of diabetes (Ishii *et al.* 1996) and inhibit the effect of vascular endothelial growth factor (VEGF) on retinal permeability and endothelial cell growth (Aiello *et al.* 1997). PKC 412 inhibits α, β, and γ isoforms of PKC which has been previously shown to inhibit ischaemia-induced angiogenesis (Seo *et al.* 1999), but early pharmacodynamic studies have revealed adverse outcomes which reflects its relative lack of specificity (Propper *et al.* 2001).

Antioxidants do block sorbitol accumulation, AGE formation and PKC activation (Nishikawa *et al.* 2000). The antioxidant tocopherol has prevented diacylglycerol and protein kinase C activation (Kunisaki *et al.* 1995), together with the haemodynamic abnormalities in diabetic rats, but in the Heart Outcomes Prevention Evaluation Study it showed a lack of effect in preventing cardiovascular risks (Pruthi *et al.* 2001).

Although the haemodynamic theory can potentially explain the vascular leakage and ischaemia, it cannot explain the neovascularization seen in proliferative retinopathy. Furthermore the basis of capillary occlusion by leukostasis occurring secondary to increased adhesion molecule (ICAM-1) expression in the diabetic state has recently been challenged by an immunohistochemical study on human tissue specimens comparing the retinal vessel expression of ICAM-1 between diabetic and non-diabetic eyes where no difference in the expression was observed. Therefore abnormalities of both blood and blood vessel wall contribute to the process of vaso-obliteration although the relative importance of either still remains to be determined (Hughes *et al.* 2004).

A further hypothesis that can explain the clinical observations has been proposed. This revolves around the role of vascular endothelial growth factor (VEGF), as an angiogenic and permeability factor together with other cytokines elaborated by the diabetic retina.

1.1.3.4 Angiogenic and Permeability Factor – VEGF

Diabetic retinopathy is essentially a disease process driven by ischaemia. Two pieces of evidence support this statement. Firstly, neovascularization of the retina, optic

nerve and iris is preceded temporally and associated spatially by retinal capillary non-perfusion (Ashton 1961). Secondly, ablation of ischaemic retina by laser photocoagulation leads to stabilization and regression of neovascularization, suggesting that such ischaemic areas may drive the neovascular process (The Diabetic Retinopathy Study Research Group 1976). These observations support the hypothesis that an angiogenic factor released by the ischaemic retina stimulates angiogenesis locally and at a distance.

A number of cytokines present in the ocular fluids have been suggested as the candidate molecule contributing to the development of angiogenesis and as a consequence to the increased vascular permeability. However this cytokine must fulfil three basic criteria. Firstly the factor must be angiogenic for endothelial cells and so stimulate proliferation and migration. Basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) fulfil this criterion (Miller *et al.* 1997). Secondly the factor should be secreted and freely diffusible so it can produce neovascularization into the vitreous and onto the iris (Miller *et al.* 1997). Since bFGF lacks a signal for secretion it is not a good candidate (Abraham *et al.* 1986). The third criterion is that the expression of this molecule should be induced by ischaemia (Ashton 1961). To date there is no evidence that IGF-1 is induced by ischaemia and that supra-physiological amounts of IGF-1 are needed to stimulate neovascularization (Grant *et al.* 1993) suggesting that this molecule may not be the major factor responsible for diabetic retinopathy. Only VEGF fits all three criteria.

VEGF is a member of a large family of angiogenic growth factors. There are six known members of the family: VEGF-A (commonly referred as ***VEGF***), placental growth factor, VEGF-B, VEGF-C, VEGF-D and VEGF-E **(see Fig 1.5)**.

VEGF-A is a heparin-binding homodimeric 45kDa glycoprotein with endothelial-specific mitogenic effects and a promoter of endothelial cell migration (Ferrara 1999). VEGF-A was initially described as a tumour-secreting peptide increasing vascular permeability (Senger *et al.* 1983) and is 50,000 times more potent than histamine in increasing dermal microvascular permeability (Aiello *et al.* 1994). It can also act as a vasodilator (Ku *et al.* 1993).

Analysis of the VEGF-A protein and gene has revealed that six different VEGF-A isoforms are derived from alternative splicing of the mRNA (**see Fig 1.5**). The smaller isoforms VEGF-A_{110}, VEGF-A_{121}, VEGF-A_{144}, VEGF-A_{165} are secreted and freely diffusible whilst the larger isoforms VEGF-A_{189} and VEGF-A_{206} are bound to heparin-containing proteoglycans on the cell surface or basement membrane (Ferrara 2000). VEGF-A itself will interact with specific endothelial-bound receptors. There are two types of VEGF receptors: high-affinity and low-affinity receptors. Both fms-like tyrosine kinase (Flt) (VEGF receptor 1) and fetal liver kinase 1 (Flk-1 / KDR)(VEGF Receptor 2) bind VEGF specifically with high affinity(Miallauer *et al.* 1993) (**see Fig 1.5**).These receptors are tyrosine kinases capable of phosphorylating other proteins in the intracellular signal transduction pathway. Two such proteins are phospholipase γ (PLC γ) and PI-3 kinase (Xia *et al.* 1996). PLC γ activation leads to increased diacylglycerol and subsequent PKC activation, particularly the β II and δ isoforms (Aiello and Hata 1999).

Studies performed *in vitro* have demonstrated VEGF-A mRNA and protein expression occurs in numerous retinal cell types including retinal pigment epithelial cells, pericytes, astrocytes, Muller cells, and endothelial cells (Miller *et al.* 1997). This suggests that VEGF-A can be produced locally, and there is substantial evidence to implicate it as the primary factor in the development of diabetic retinopathy and macular oedema.

In vitro (Shima *et al.* 1995) and *in vivo* (Miller *et al.* 1994) work has confirmed increased production of VEGF in ischaemic retinal cells. Miller *et al.* demonstrated a temporal and spatial relationship of VEGF-A production with iris neovascularization

in a monkey model (Miller *et al.* 1994). Intravitreal sustained release of VEGF-A using a pellet containing the cytokine produces vasodilatation, retinal tortuosity and

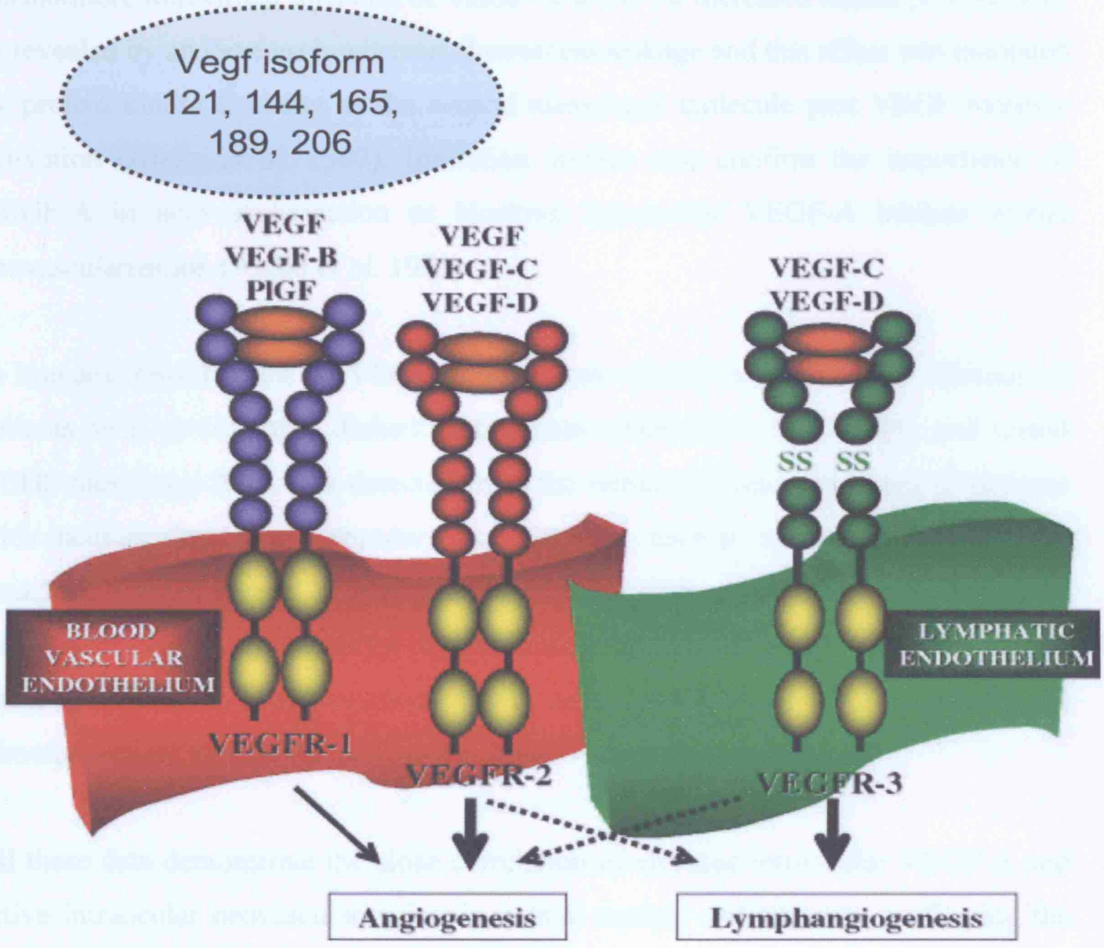


Fig 1.5

Figure 1.5 VEGF Family. Schematic diagram of the VEGF family, VEGF-A (VEGF) isoforms and the VEGF receptors with the associated targeted tissues (reprinted from (Tammela et al. 2005).

retinal oedema, capillary occlusion with ischaemia, microaneurysms, iris and retinal neovascularization and neovascular glaucoma, all characteristic findings of diabetic retinopathy in a cynamologous monkey (Tolentino *et al.* 1996), (Tolentino *et al.* 1996). Electron immunocytochemical staining in the primate model demonstrated breakdown of the blood retinal barrier with a pellet containing 100μg of VEGF-A. Furthermore intravitreal injection of VEGF-A in the rat increased retinal permeability as revealed by an increase in vitreous fluorescein leakage and this effect was mediated by protein kinase C acting as the second messenger molecule post VEGF receptor activation (Aiello *et al.* 1997). Inhibition studies also confirm the importance of VEGF-A in neovascularization as blocking intraocular VEGF-A inhibits retinal neovascularization (Aiello *et al.* 1995).

In humans, raised levels of VEGF-A have been shown to occur in the vitreous of patients with proliferative diabetic retinopathy (Malecaze *et al.* 1994) and raised VEGF messenger RNA was detected from the retinas of enucleated eyes of patients with neovascularization secondary to diabetes, central vein occlusion, retinal detachment and tumours in a post-mortem study (Pe'er *et al.* 1995). Furthermore clinico-pathological correlations demonstrated that VEGF-A in the vitreous of patients with active neovascularization was capable of binding VEGF receptors and stimulate retinal endothelial cell growth *in vitro* (Aiello *et al.* 1994).

All these data demonstrate the close correlation of elevated intraocular VEGF-A and active intraocular neovascularization in animal models and humans, confirming the importance of VEGF-A in this disease process.

There is also evidence suggesting VEGF-A is able to induce increased vascular permeability. In animal models exogenous VEGF-A can produce retinal oedema (Ozaki *et al.* 1997) by BRB breakdown of the capillaries and venules of the superficial inner retinal vasculature (Qaum *et al.* 2001). Studies on streptozotocin-induced diabetic rats revealed an increase in VEGF-A mRNA levels compared to control animals especially in the ganglion cell layer and the inner nuclear layer (Gilbert *et al.* 1998). Further, in this animal model the rate of both blood retinal barrier (BRB) breakdown, as demonstrated by albumin immunohistochemistry, and VEGF immunoreactivity increased in proportion to the duration of the diabetes. In

addition the rate of BRB breakdown was higher for vessels with VEGF-A immunoreactivity compared to vessels without it (Murata *et al.* 1996).

These animal studies suggest an intimate relationship between increased vascular permeability in the diabetic retina and VEGF-A expression. However a *post-mortem* study on diabetic eyes with background retinopathy and non-diabetic control eyes showed VEGF-A expression as described in the animal work, but its abundance and distribution was not altered when compared to the non-diabetic eyes (Gerhardinger *et al.* 1998) . However, this may be due to hypoxia-induced VEGF-A subsequent to circulatory collapse in these patients and these data are therefore inconclusive.

The factors controlling VEGF-A production are complex. Tissue hypoxia is known to upregulate VEGF-A by increasing both the expression and stability of VEGF-A mRNA (Levy *et al.* 1996). The mechanism by which other cytokines, AGE and oxidative stresses produce transcriptional events leading to VEGF-A upregulation is unclear. However oxidative stresses have been found to increase VEGF-A through a mechanism involving *s*ignal *t*ransducer and *a*ctivator of *t*ranscription factor 3 (STAT 3) (Bartoli *et al.* 2003). STAT proteins are latent cytoplasmic transcription factors that regulate gene expression induced by cytokines, interferons and growth factors. STAT 3 is activated by reactive oxygen species and inflammatory mediators and its activation has been correlated with increased rates of VEGF-A expression and angiogenesis *in vivo* and *in vitro* (Niu *et al.* 2002).

1.1.3.5 VEGF and the other major existing theories

In relation to the metabolic theory a number of studies have suggested close links between components of that theory and VEGF. Both glucose (Tilton *et al.* 1997) and AGE (Lu *et al.* 1998) stimulate VEGF-A gene expression and high glucose concentrations can induce VEGF-A protein expression through a PKC mediated pathway whilst AGE increases VEGF-A expression through a reactive oxygen intermediate-mediated pathway, mainly by increasing VEGF-A mRNA stability (Lu *et al.* 1998).

The link between VEGF and the haemodynamic theory is that VEGF can act as a

vasodilator and can induce leukostasis via the induction of ICAM-1(Miyamoto *et al.* 2000). Also diabetes has been shown to produce inflammatory mediators via the cyclo-oxygenase pathway in the rat retina (Ayalasomayaajula and Kompella 2003). Products of cyclooxygenase-mediated arachidonic acid metabolism (prostaglandins, prostacyclin and thromboxane) play a key role in ocular inflammation and are able to induce VEGF expression *in vitro* (Cheng *et al.* 1998). VEGF induces prostacyclin formation in cultured endothelial cells and ICAM-1 expression.

Therefore the metabolic and haemodynamic components may exert their influence towards the development of macular oedema through VEGF (**see Fig 1.6**).

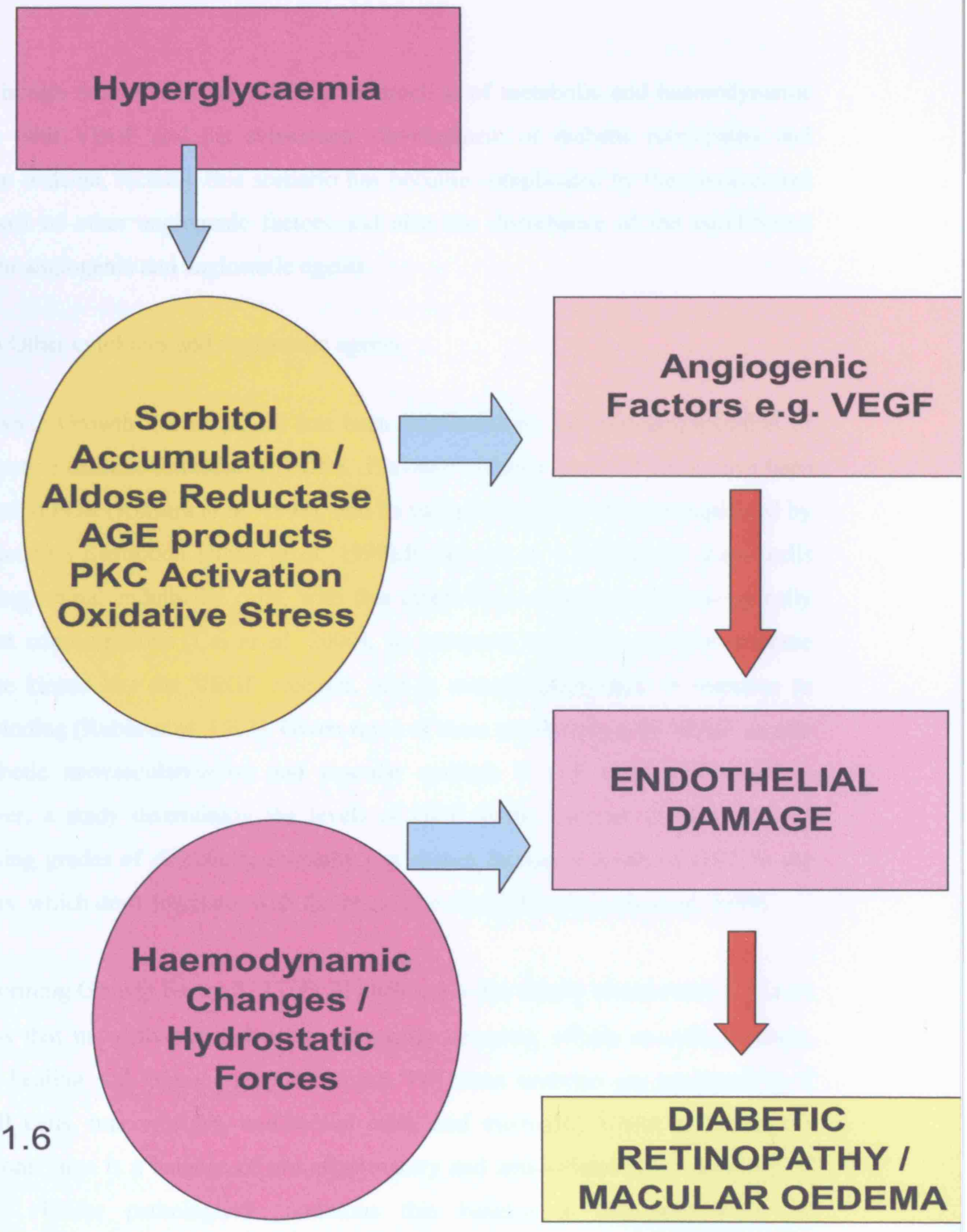


Fig 1.6

Figure 1.6 Potential Consequences of the Metabolic Alteration in Diabetes Mellitus

Even though there exists this potential interaction of metabolic and haemodynamic factors with VEGF and the subsequent development of diabetic retinopathy and macular oedema, recently this scenario has become complicated by the involvement of a host of other angiogenic factors and also the disturbance of the equilibrium between angiogenic and angiostatic agents.

1.1.3.6 Other cytokines and angiostatic agents

Hepatocyte Growth Factor (HGF) has been implicated in the neovascularization of proliferative diabetic retinopathy (PDR). Elevated vitreous levels of HGF have been reported in PDR (Katsura *et al.* 1998), and its vasopermeability effect is explained by tight junction disruption (Jiang *et al.* 1999).It can act as a mitogen to many cells including retinal endothelial cells, with this effect also occurring at physiologically relevant concentrations (Cai *et al.* 2000). Its receptor, c-Met, is a transmembrane tyrosine kinase like the VEGF receptor, and is autophosphorylated in response to HGF binding (Rubin *et al.* 1993). Given some of these similarities with VEGF its role in diabetic neovascularization and macular oedema is still under investigation. However, a study determining the levels of HGF in the aqueous of patients with increasing grades of diabetic retinopathy has shown increased levels of HGF in the aqueous, which does correlate, with the grade of retinopathy (Shinoda *et al.* 1999).

Transforming Growth Factor β1 (TGF β1) belongs to the family of structurally related proteins that have diverse and often apparently opposing effects on inflammation, wound healing and immune responsiveness. The three isoforms are produced by T cells, B cells, macrophages, endothelial cells, and microglia. Under physiological conditions there is a balance of proinflammatory and anti-inflammatory activities of TGF β. Under pathological conditions this balance is disrupted with the proinflammatory functions promoting recruitment of white cells, though as inflammation procedes it exerts an apoptotic effect on the white cells. It also then promotes the deposition of extracellular matrices, including fibronectin, collagen and proteoglycans (Hofman and Hinton 2001. In particular relation to the retina itself it can modulate increased vascular permeability in retinal endothelial cells {Behzadian *et al,* 2001) and is known to have a regulatory role in angiogenesis (Pepper 1997).

Matrix Metalloproteinase 9 (MMP 9) belongs to a group of enzymes that are involved in numerous physiological and pathological processes associated with extracellular matrix remodelling. In cell culture models MMP9 can disrupt the tight junctions between retinal endothelial cells (Behzadian *et al.* 2001), and its levels are increased in the vitreous of patients with diabetic retinopathy compared to controls (Jin *et al.* 2001). Matrix Metalloproteinase 9 (MMP 9) belongs to a group of enzymes involved in extracellular matrix remodelling and may increase vasopermeability. In cell culture it disrupts retinal endothelial cell tight junctions (Behzadian *et al.* 2001; Harkness *et al.* 2000) and vitreous levels are increased (Jin *et al.* 2001).

The angiopoietins are endothelium specific growth factors that work in concert with Vascular Endothelium Growth Factor (VEGF) to modulate the processes of physiological angiogenesis and pathological neovascularization (Adamis *et al.* 1996; Sato *et al.* 1995). There are four known angiopoietins (Ang 1 to Ang 4) (Koh *et al.* 2002). Both Angiopoeitin 1 (Ang 1) and Angiopoietin 2 (Ang 2) have been extensively studied in animal and in vitro models particularly in relation to diabetic retinopathy. Ang 1 has been shown to promote endothelial cell survival without causing proliferation (Kim *et al.* 2000), to stabilise endothelial interactions with surrounding support cells (Thurston *et al.* 1999), to block the vascular permeability effects of VEGF *in vivo* (Thurston *et al.* 1999) (Thurston *et al.* 2000), and to suppress the development of diabetic retinopathy and reduce both vascular endothelial injury and blood-retinal barrier breakdown in a rat diabetic model (Joussen *et al.* 2002). In contrast, Ang 2 is expressed at sites of vascular remodelling in the angiogenic process (Maisonpierre *et al.* 1997) and is upregulated by hypoxia, VEGF (Mandriota and Pepper 1998), and retinal ischaemia (Oh *et al.* 1999).

To counterbalance the effect of pro-angiogenic agents, like VEGF, there are naturally occurring angiostatic agents in the human eye, particularly the recently identified 50KDa protein derived from retinal pigment epithelium called pigment epithelium-derived factor (PEDF). It has been shown to be a more potent inhibitor of angiogenesis than angiostatin, thrombospodin-1 and endostatin (Dawson *et al.* 1999), the other naturally occurring angiostatic agents found in the human eye. Soluble Flt-1 receptor, derived from the flt-1 gene, is another important anti-angiogenic agent. This flt-1 gene encodes for two proteins. One which is the full length version and is the

transmembrane VEGF receptor 1; whilst the second protein is formed by the premature termination of the flt-1 mRNA because of an aberrant polyadenylation in intron-13 (Shibuya 2001)which then produces and releases the extracellular domain of the recptor which is soluble in the extracellular space and vitreous. In this form with its extracellular- VEGF binding domain free in the vitreous it can inhibit VEGF and has been shown to prevent angiogenesis (He *et al.* 1999). However the role of both these anti-angiogenic agents and their relationship with VEGF in the different grades of diabetic retinopathy and macular oedema is still to be determined (**see Fig 1.7**).

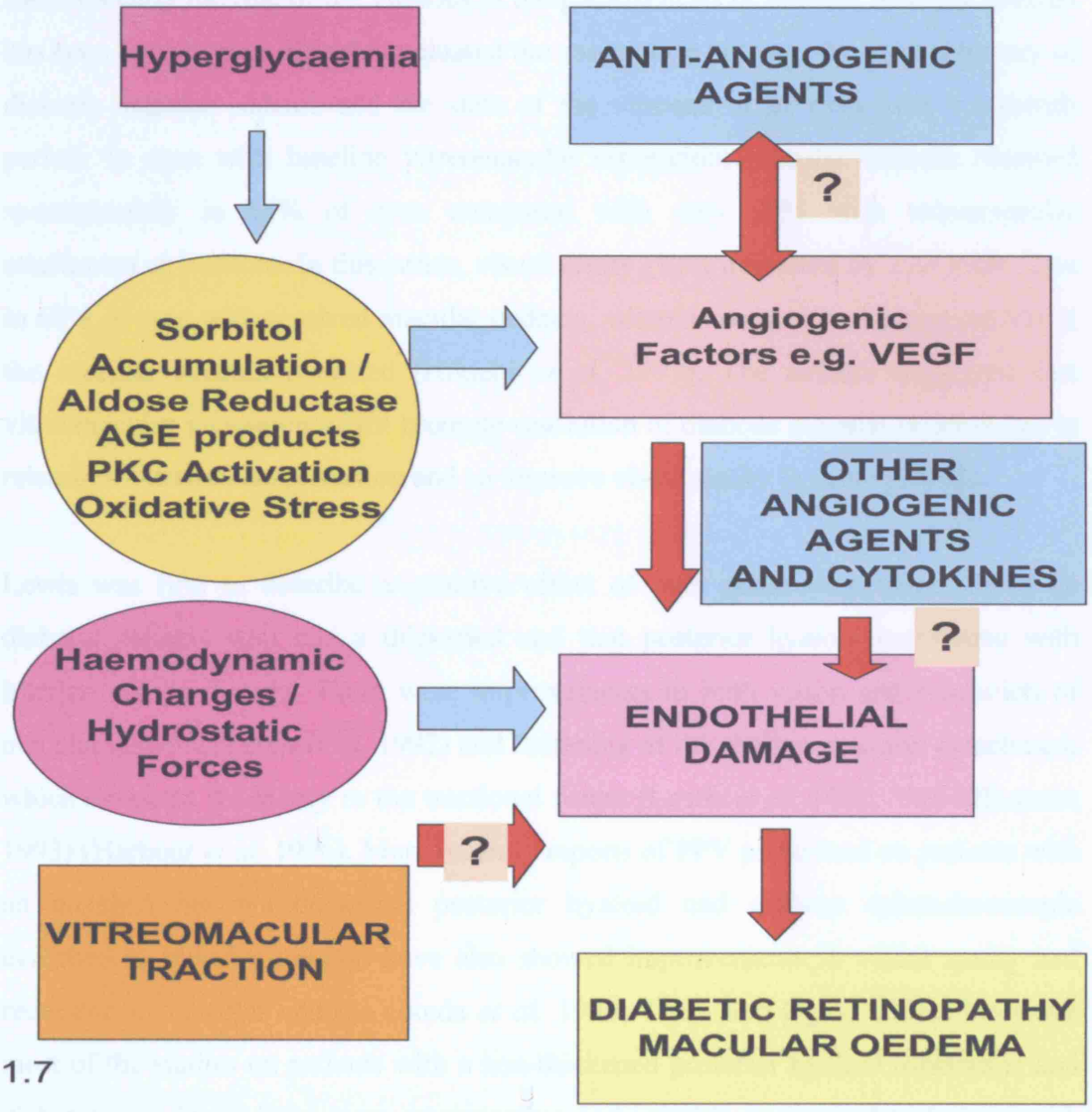


Fig 1.7

Figure 1.7 Potential Consequences of the Metabolic Alteration in Diabetes Mellitus with Interaction of Tractional Forces and Anti Angiogenic Agents.

Despite such elaborate evidence to suggest an active role of these growth factors and cytokines as outlined above in the development of DMO, there is little evidence in the literature of their levels in the ocular fluids of patients with macular oedema and so of their influence on the blood retinal barrier. This is in direct contrast to published data for patients with proliferative diabetic retinopathy (PDR), particularly in relation to levels of VEGF.

1.1.3.7 Mechanical / Tractional Forces

More recently the role of the vitreous in the pathogenesis of diabetic macular oedema has been investigated. Hikichi evaluated the association between the natural history of diabetic macular oedema and the state of the vitreous in 82 eyes over a 6-month period. In eyes with baseline vitreomacular separation, macular oedema resolved spontaneously in 55% of eyes compared with only 25% with vitreomacular attachment at baseline. In this series, visual acuity (VA) improved by 2 or more lines in 63% of eyes with resolved macular oedema, whereas no eyes had improved VA if the macular oedema persisted (Hikichi *et al.* 1997). The authors suggested that vitreomacular separation might promote resolution of diabetic macular oedema due to release of vitreomacular traction and so improve visual acuity in some patients.

Lewis was first to describe a positive effect of pars plana vitrectomy (PPV) on diabetic patients who had a thickened and taut posterior hyaloid membrane with traction on the macula. There were improvements in both vision and resolution of macular oedema (Lewis *et al.* 1992) and flattening of the shallow macular detachment which develops secondary to the tractional forces (Lewis *et al.* 1992; Van Effenterre 1993) (Harbour *et al.* 1996). More recently reports of PPV performed on patients with an attached but non-thickened posterior hyaloid and without ophthalmoscopic evidence of macular traction have also showed improvements in visual acuity and reduction of macular oedema (Ikeda *et al.* 1999; Tachi and Ogino 1996). However most of the studies on patients with a non-thickened posterior hyaloid membrane and diabetic macular oedema were retrospective and variable in surgical technique with some patients having cataract (Tachi and Ogino 1996) or internal limiting membrane (Gandofer *et al.* 2000) removed simultaneously.

The pathological basis for this clinical work is that in diabetes the vitreous undergoes abnormal glycation of its collagen and this abnormal collagen structure can destabilize the vitreous, leading to traction on the macula (Sebag and Boulazs 1984). The increased vascular permeability then allows for a pool of chemoattractant factors and agents which promotes cell migration to the posterior hyaloid (Sebag 1996). These cells may then contract producing macular traction, a shallow macular detachment and exacerbation of macular oedema. (Jumper *et al.* 2000).

Therefore in the development of macular oedema, tractional forces from the vitreous onto the macular surface may contribute to the vascular damage, promoting further and exacerbating increased vascular permeability. However the significance and importance of this component in the pathogenesis of diabetic macular oedema still remains uncertain in patients without the taut posterior hyaloid face (representing a minority of cases), as does the relationship between traction and angiogenic / anti-angiogenic factors (**see Fig 1.7**).

1.1.4 Treatment of Diabetic Retinopathy

Evidence-based treatment of diabetic retinopathy involves indirect management with optimizing glycaemic control and systemic hypertension and directly with argon photocoagulation.

1.1.4.1 Optimizing glycaemic control and systemic hypertension

Two important multicentre clinical trials, the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS), conclusively demonstrated that intensive glycaemic control slows the onset and progression of diabetic retinopathy and other vascular complications in both type 1 and type 2 diabetes (The Diabetes Control and Complications Trial Research Group 1993; UK Prospective Diabetes Study (UKPDS) Group 1998).

DCCT showed that based on a total of 1,441 patients with type 1 DM followed up over 6.5 years that with intensive glycaemic control there is a 76% reduction in the onset of DR, a 63% reduction in the progression of DR, a 47% reduction in the

development of severe nonproliferative or proliferative DR, a 26% reduction in the development of macular oedema, and a 51% reduction in the overall need for laser. However the disadvantages of intensive insulin therapy induced increased episodes of hypoglycaemia and a two times greater occurrence of early worsening of diabetic retinopathy, which was reversed after 18 months. UKPDS studied 3,867 patients with newly diagnosed type 2 DM who were randomly assigned to intensive or conventional control. Intensive glycaemic control resulted in a 17% reduction in the progression of retinopathy and a 29% reduction in the overall need for laser.

Patients with diabetes have a higher prevalence and incidence of systemic hypertension than people without diabetes. It has also been demonstrated that elevated blood pressure (BP) is associated with more severe levels of retinopathy and increased risk of progression of retinopathy when compared to diabetic patients without hypertension (Marshall *et al.* 1993). UKPDS has demonstrated that of 1148 patients who were randomised to tight or conventional BP management and followed up for 8.4 years, there was a 34% reduction in DR progression and 47% reduction in visual acuity loss in the patients randomised to tight BP control and this risk reduction was independent of glycaemic control.

1.1.4.2 Argon laser photocoagulation

Current treatment is based on a series of clinical trials. The main trials include Diabetic Retinopathy Study (DRS) and the Early Treatment Diabetic Retinopathy Study (ETDRS).

The Diabetic Retinopathy Study demonstrated that scatter (panretinal) laser photocoagulation significantly reduces the risk of severe visual loss (best corrected visual acuity of 5 / 200 or worse) in patients with proliferative diabetic retinopathy (The Diabetic Retinopathy Study Research Group 1976).

The treatment of diabetic macular oedema was initially studied in four early trials Patz *et al.* (1973), British Multicentre Study Group (1983), Blackenship (1979) and Olk (1986). In these four trials one eye was treated with argon laser, the fellow eye acted as control and in the majority of cases bilateral macular oedema without PDR

was present. Even though the numbers of patients in these four trials were small and the admission criteria different, the major results showed a statistically significant beneficial treatment effect. The untreated eyes lost 2 or more lines of VA over the 2 year follow up period in comparison to the treated eye.

However, it is the multicentre randomized prospective ETDRS trial, which is considered the landmark study, and its clinical recommendations have been widely adopted.

The Early Treatment Diabetic Retinopathy Study Research Group (1985) (ETDRS) formally addressed the issue of treatment for diabetic macular oedema by conducting a large long-term randomized control trial. From April 1980 to August 1985, 3,928 patients with diabetes mellitus with early proliferative retinopathy, moderate to severe nonproliferative retinopathy and / or diabetic macular oedema in each eye were recruited. Patients were either randomised into deferral of treatment or immediate treatment with the immediate group further randomised into immediate focal or panretinal photocoagulation plus follow-up focal. They described three types of lesions angiographically and treated each with focal photocoagulation: focal leaks such as microaneurysms located at least 300 μm from the foveal centre; diffuse leaks and avascular zones other than the normally present FAZ. All treatable lesions were located within 2 disc diameters of the foveal centre. Focal treatment to microaneurysm sites received 50-100 μm argon blue-green or green-only burns of 0.1 sec duration or less, with adequate power to obtain definite whitening around the microaneurysm. Areas of diffuse leakage or nonperfusion were treated with a grid pattern. The goal of treatment in such cases was to produce a burn of light to moderate intensity, not more than 200 μm in diameter. To accomplish this, a 50-200 μm spot size was utilized. A space one burn wide was left between each lesion. Burns were also placed in the papillomacular bundle, but not closer than 500 μm from the centre of the fovea (Early Treatment Diabetic Retinopathy Study Research Group 1985).

At 1 year follow-up for patients with clinically significant macular oedema, 5% of treated eyes versus 8% of deferred eyes lost more than 15 letters on the ETDRS chart which is equivalent to doubling of the visual angle i.e. MVL, moderate visual loss. At

the 3-year follow-up, the figures were 12% and 24% respectively (Early Treatment Diabetic Retinopathy Study Research Group 1985). ETDRS did not publish the results of re-treatment and therefore although repeated focal treatment is often undertaken, the efficacy remains unproven.

Given the clinical work which does confirm the efficacy of argon laser treatment for macular oedema despite the known side effects of treatment there are a number of potential mechanisms which have been described to explain its mode of action on oedematous tissue and the resolution of that oedema. In a study on 46 eyes (34 patients) with macular oedema, in the patients where there was resolution of the oedema, this was paralleled by a decrease in the passive permeability of the retinal vasculature using fluorescein as a tracer molecule (Sander *et al.* 2002). Other mechanisms, which have been proposed, fall into three main categories. These are reduction in the oedema causing pathology, modification of physical factors that favour oedema and improvement of water absorption.

Argon laser treatment is thought able to reduce the pathology causing oedema by improving the retinal oxygenation, which would reduce ischaemia and metabolic damage, by destroying leaky retinal, and choroidal vessels and damaging RPE cells which stimulates RPE healing. The process of healing stimulates the release of reparative factors and other cytokines e.g. PEDF and lessen the release of inflammatory and cytotoxic factors. Laser is also proposed to open the RPE barrier and so improve water and protein egress from the subretinal space, it may alter Bruch's membrane and so allow for the dissipation of lipids, which prevent water and protein absorption and may stimulate the clearance of intraretinal protein by phagocytosis. These proposed physical changes are thought to modify the physical microenvironment that favours the retention of solutes and fluid within the retina. Lastly, laser treatment may promote improvement of water absorption by facilitating passive water absorption into the choroid as confirmed by Sander et al as described above, and promote new and healthy RPE cells which are then able to restore metabolic conditions that favour outward water transport. (Marmor 1999)
Even though laser is thought to promote oedema resolution by these mechanisms and despite exemplary laser treatment according to ETDRS guidelines, 50% of patients show doubling of the visual angle i.e. MVL ie loss of vision. Therefore focal

treatment does not prevent visual loss in all cases or rarely improves vision in all cases. Furthermore patients with associated systemic diseases particularly renal failure and hypertension have an increased risk of a poor outcome, if these factors cannot be adequately controlled. Also ETDRS did not include a sub-analysis of patients treated predominantly with either focal alone (where focal laser is applied to microaneurysms) or grid alone (where laser treatment is applied in a grid pattern over areas of retinal thickness or capillary nonperfusion) but whilst focal laser treatment appears to be efficacious, the results of grid treatment have been disappointing (Bailey *et al.* 1999).

Furthermore laser treatment itself can produce potential side effects, including reduction in visual field, nyctalopia, damage to other intraocular structure, misplaced burns, and the resultant chorioretinal scars produced by the laser burns can enlarge over time resulting in foveal encroachment, photoreceptor loss, pigment clumping and vessel attenuation (Aiello *et al.* 2004).

Given these problems, and that laser treatment at best is able to preserve vision rather than improve vision, which has been damaged by macular oedema, a search for an alternative or additional approach is ongoing. To deliver such an alternative approach would first need an understanding of macular oedema and the structural mechanisms which when maintained keep the retina free of fluid accumulation but when an insult e.g. pro-angiogenic or hydrostatic forces are applied, dynamic changes and processes begin within this structural substrate leading to macular oedema. In effect primary understanding of the blood- retinal barrier is needed.

CHAPTER 1.2. RETINAL STRUCTURE

1.2.1 Retina Structure

The retina is divided into peripheral retina and central retina or macula lutea. The macula is that portion of the posterior retina that contains xanthophyllic pigment and two or more layers of ganglion cells. It measures 5.5 mm in diameter and is centred 4 mm temporal and 0.8 mm inferior to the optic disc **(see Fig 1.8).** Microscopically the macula can be divided into several zones. The fovea (fovea centralis) is a depression in the inner retinal surface in the centre of the macula and measures 1.5 mm or one disc diameter in size. The central floor of the fovea is the foveola (0.35 mm diameter) and lies within the capillary free zone called the ***Foveolar Avascular Zone*** (FAZ) **(see Fig 1.9).** A small depression in the foveola is called the umbo **(see Fig 1.10). (Figs 1.8 to 1.10 have been reprinted from** (Gass 1997)).

The retina is a delicate transparent tissue of maximal thickness (about 0.55 mm) at the foveal margin and minimal thickness (about 0.13 mm) at the umbo. It is a multilayered structure composed of neuronal, glial, epithelial and endothelial cells. In total there are 10 layers, seven of which are neural (Fine and Yanoff 1972). These layers form one single compartment with two vascular layers supplying the retina but separated from the retina by the blood retina barrier. This description is based on light microscopy and horseradish peroxidase diffusion studies, which demonstrated no continuous barriers to fluid movement within the retina apart from the blood retina barrier (Peyman and Bok 1972). However clinically disruption of the inner blood retina barrier (as in diabetes mellitus) leads to intraretinal oedema but does not produce serous retinal detachment as would be predicted if the retina was one single compartment with no barriers to movement of fluid. Conversely disruption of the outer blood retina barrier as in central serous chorioretinopathy producing serous retinal detachment does not also produce intraretinal oedema.

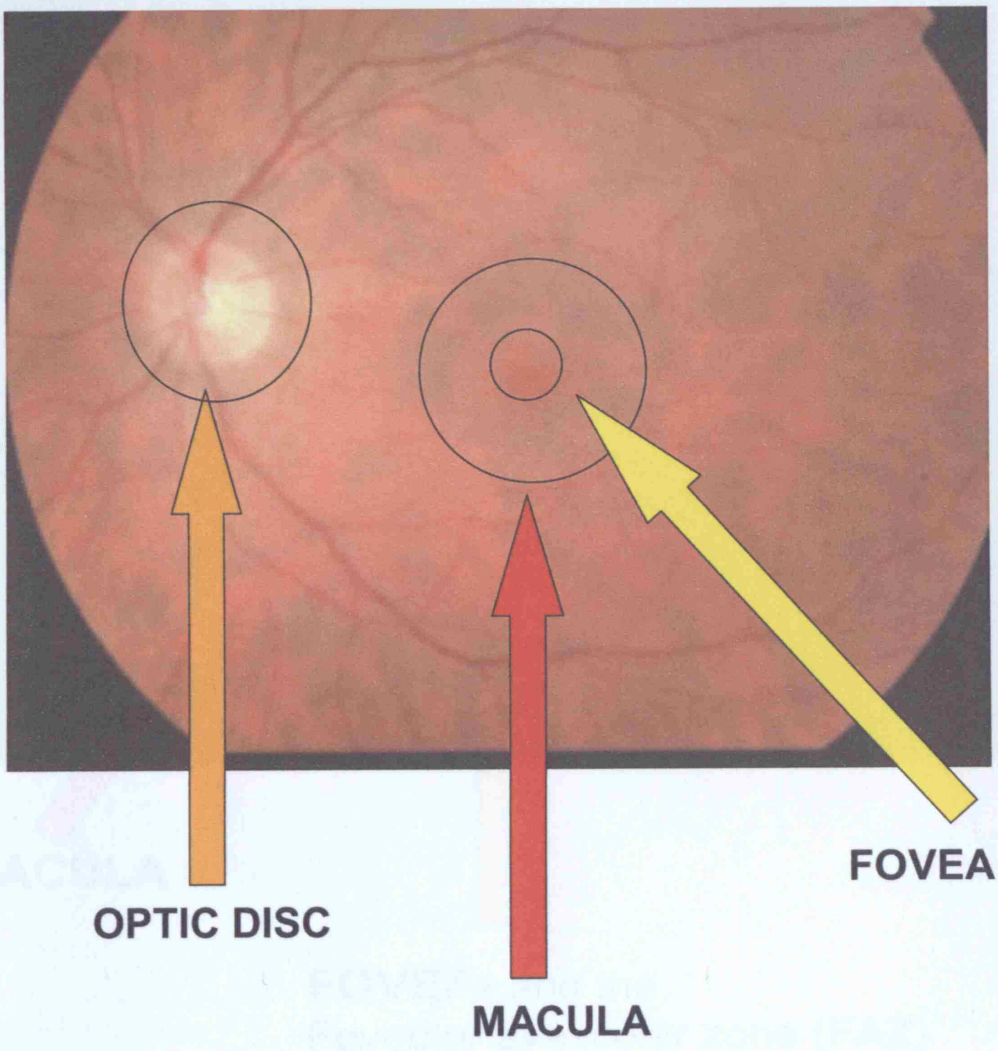


Fig 1.8

Figure 1.8 The Posterior Pole.

Normal posterior pole, fovea and macula.

NORMAL RETINAL VASCULATURE AND THE FOVEOLAR AVASCULAR ZONE

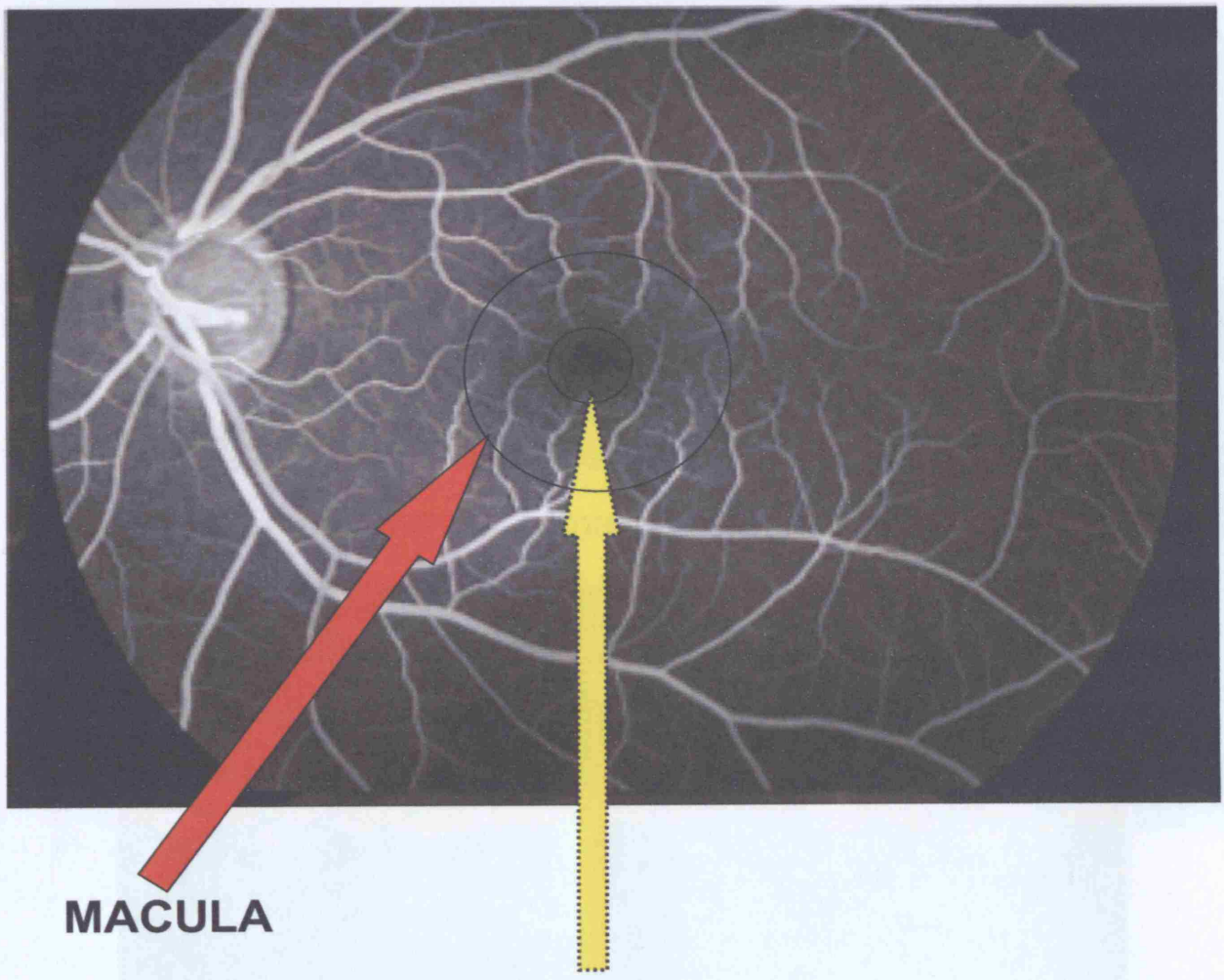
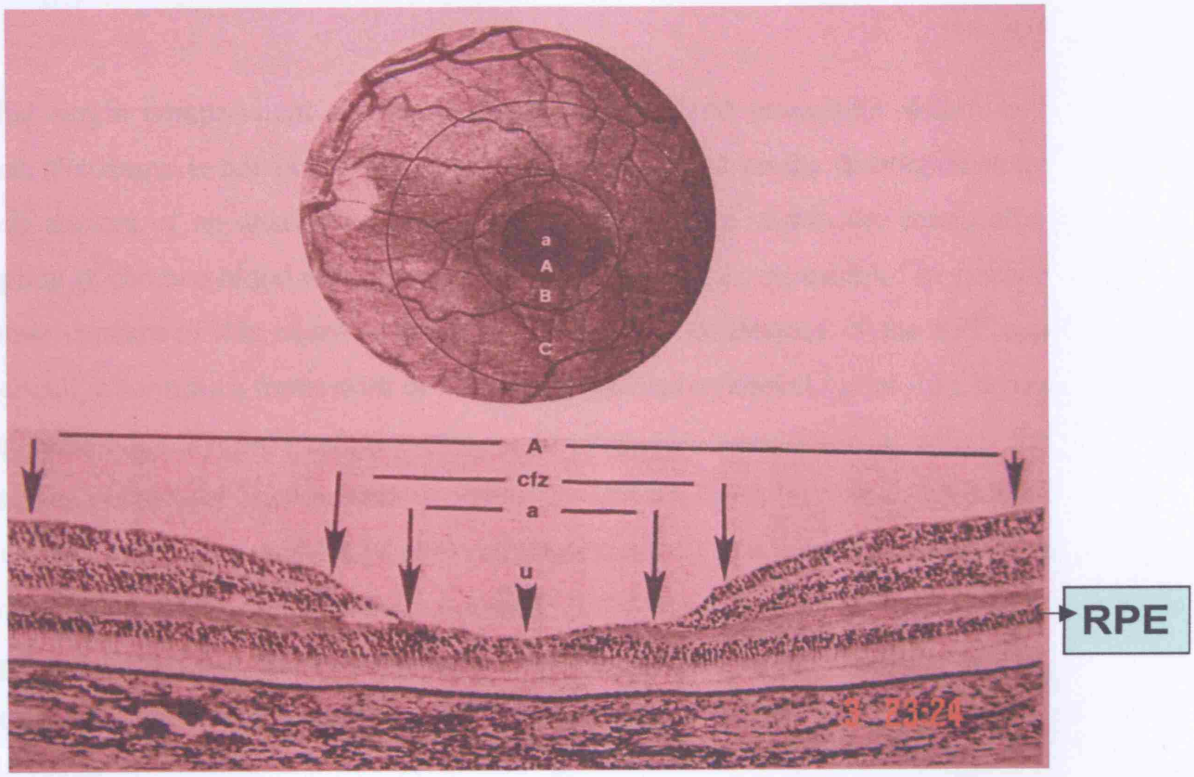


Fig 1.9

Figure 1.9 The retinal vasculature and the foveolar avascular zone as seen on fluorescein angiography.

Topographic anatomy (above) and histology (below) of the macula



Corresponding OCT image of the macular area

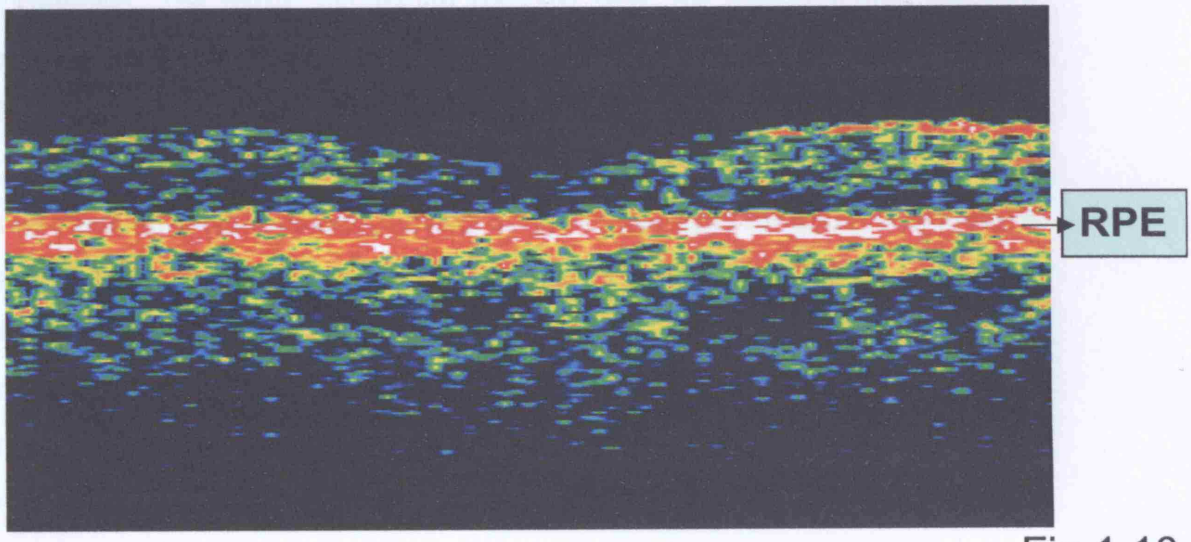


Fig 1.10

Figure 1.10 Comparison of Macula Histology and Topography with OCT imaging. The first image was reprinted from ' Stereoscopic Atlas of Macular Diseases diagnosis and treatment' Volume 1, 4th Edition by J Donald M. Gass

As this single compartment model of understanding fluid movement within and beneath the retina is not borne out clinically, this has lead to the development by various authors of an alternative model of fluid movement within the retina after disruption of the two blood retina barriers by differing disease processes. In essence the basic concept of this alternate model highlights the importance of the RPE and Muller cell in forming a framework of two monostratified epithelial / glial -like layers facing each other by their apical surfaces, forming two compartments within the retina. The outer layer is composed of RPE cells and the inner layer is composed of Muller cells. Like other epithelial / glial cells they have villi on their apical surface, a basement membrane and inter-cellular junction complexes on the lateral surface (tight and gap junctions). Also like other epithelial / glia tissue, they are primarily avascular, contacting the blood supply via their basal surface (Sisak *et al.* 2004).

Two main circulations supply the retina: the retinal capillaries and the choroid and choriocapillaris. The retinal circulation (derived from the central retinal artery) is composed of a plexus in the ganglion cell layer and from two plexi in the superficial and deep layers of the inner nuclear layer. These three plexi meet around the fovea to form a vascular arcade and a central avascular zone (the Foveolar Avascular Zone). The outer retina (photoreceptors), having a high oxygen and metabolic requirement, are supplied by the choriocapillaris. The inner retina to the level of the outer plexiform layer is supplied by the retinal capillaries (Krebs and Krebs 1991). The watershed zone between the retinal and choroidal circulations appears to be at the photoreceptor cell level. The photoreceptor has components in both compartments. These vascular networks are separated from the cellular elements by two blood retinal barriers.

1.2.2. The Two Retinal Compartments (**Fig 1.11**)

The outer compartment extends between the outer limiting membrane (OLM) and the RPE basement membrane. It is supplied by the choriocapillary network through the outer BRB formed by the RPE. The main neural elements are the inner and outer segments of the photoreceptor cell. The main metabolic activity of this compartment

is the control of the retinol cycle by the RPE (Bok 1988) and concerned with the transduction of photic energy into neuronal electrical signals (Singelman and Ozanics 1990). This requires a vast oxygen and metabolite supply as well as mobilization of large quantities of heat. Therefore the choroidal capillaries are short, wide, fenestrated and of high volume. Breakdown of the outer barrier leads to serous retinal detachment and given that this compartment is extensive large amounts of fluid can accumulate in the potential inter-photoreceptor space without damaging the compartment borders.

The inner compartment extends between the OLM and the Muller cell end-feet and their basal membrane, the internal limiting membrane (ILM). It is supplied by the central retinal artery, with the endothelial cells of retinal capillaries forming the inner BRB. The epithelial cell is the Muller cell with the principal neural elements being the photoreceptor, bipolar and ganglion cell and their interconnections. The main metabolic task is related to the electrical and biochemical activity of the neural elements and this activity depends on oxygen supply. Therefore the retinal capillaries are long and narrow and non fenestrated. This arrangement allows for an intimate contact between erythrocytes and the capillary wall which allows for maximal oxygen transfer. Breakdown of the inner barrier leads to retinal oedema to the level of the OLM. As this compartment is not extensive, fluid accumulation will compromise the neural elements: chronic accumulation proceeds to degeneration of the neural elements.

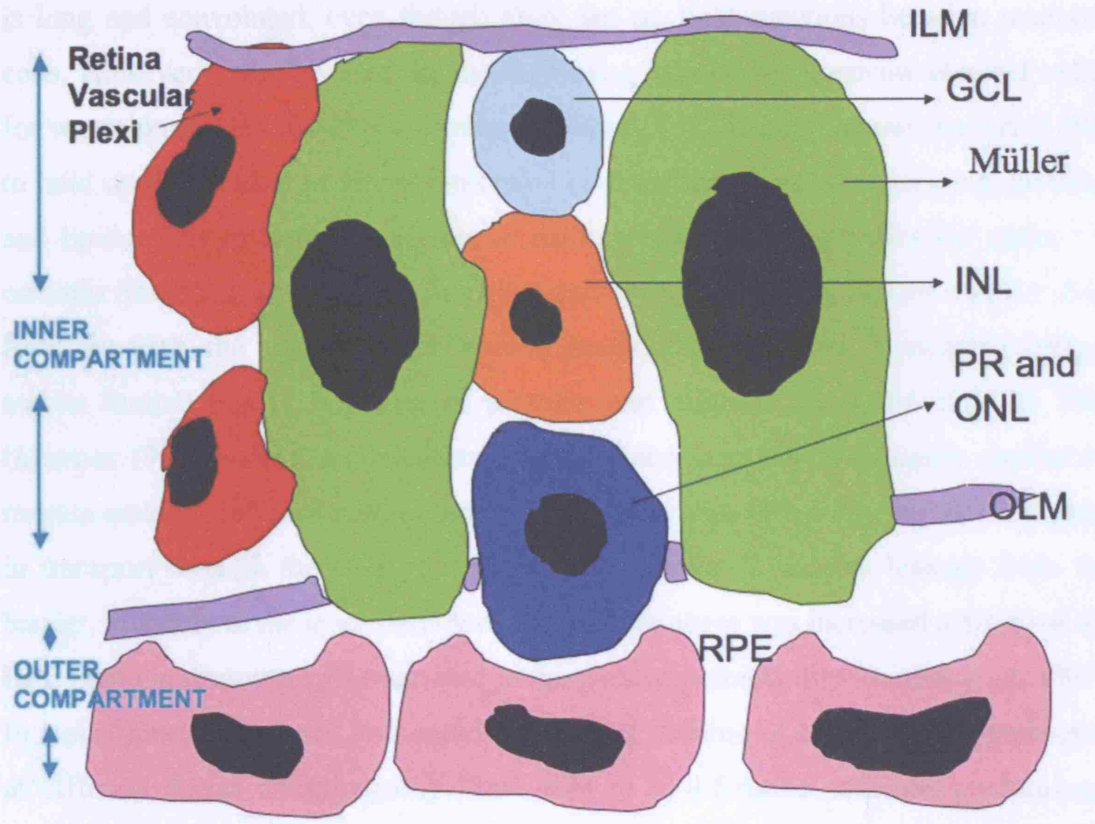


Fig 1.11. The two retinal compartments.

ILM= Internal Limiting Membrane, GCL=ganglion cell layer, INL= inner nuclear layer, PR and ONL= photoreceptors and outer nuclear layer, RPE= retinal pigment epithelium

The OLM is a semi permeable barrier separating the two compartments. It is formed by the continuous sheet of gap junctions that connect the external end-feet of the Muller cell with the proximal part of the photoreceptor inner segment as they emerge from the cell body. The OLM is not a barrier like the BRB because it contains gap junctions and also provides a mechanical function and this maybe one of the factors underlying the isolation of many inner compartment pathologies e.g. cystoid oedema, retinal haemorrhages and exudates.

Therefore retinal tissue itself impedes the free flow of water (Fatt and Shantinath 1971) because the interstitial pathway from the vitreous to the subretinal space (SRS)

is long and convoluted, even though there are no tight junctions between neuronal cells. However at the level of the outer limiting membrane, a narrow channel exists for water to progress towards the subretinal space (SRS). This narrow channel is able to hold up the passage of larger molecules entering the interstitial space e.g. proteins and lipids. This in turn is then able to retain water within the interstitial space via osmotic forces. Therefore, even though a powerful RPE pump exists to remove fluid form the SRS, the extent of its ability to perform this function in macular oedema maybe limited due to build up of proteins and minimal water entering the SRS (Marmor 1999). Indeed, a clinical study of 25 diabetic eyes with clinically significant macula oedema and moderate to severe retinopathy concluded that the major change in transport through the BRB was due to an increase in passive leakage from the barrier, probably at the inner BRB level. In contrast there was increased activity of the RPE pump in response to the increase in the passive permeability (Sander *et al.* 2001). In a post-mortem study of immunohistochemical staining of albumin in diabetic eyes at different stages of retinopathy from mild to proliferative confirms predominant leakage from the inner retinal barrier and only minor leakage from the outer barrier once proliferative retinopathy has ensued(Vinores *et al.* 1990).

1.2.3 Blood-Retinal Barrier (BRB)

The blood retina barrier consists of complexes of tight junctions (*zonula occludens*) between the cells of the endothelium or epithelium that prevents the passage of larger molecules (e.g. serum proteins) or particles through the extracellular clefts of the cellular sheath. There are two barriers, one for each circulation. The outer barrier consists of tight junctional complexes between the RPE cells; the inner barrier contains tight junctions between the endothelial cells lining the retinal capillaries. Furthermore, processes from the glial Muller cell also wrap the retinal capillaries in the INL in the macula with the corresponding capillaries in the GCL in close contact with astrocytic processes. These glial cells secrete factors which either enhance (e.g. glial cell line-derived neurotrophic factor (Igarashi *et al.* 2000) or decrease the tightness of the barrier provided by the vascular endothelium (e.g. VEGF) (Aiello *et al.* 1995). The BRB also has a functional component consisting of the *RPE pump* that removes water from the sub-retinal space into the choriocapillaris.

1.2.3.1 Intercellular clefts and Junctional Proteins.

Multicellular systems form an assembly of cellular sheets that separate compartments of different composition. Epithelial and endothelial cells form these sheets and the barrier function is achieved when these cells adhere to each other forming different cell-to–cell junctions. Tight junctions (zonula occludens) are the most apical junctions between epithelial cells but not necessarily between endothelial cells. The other ubiquitous junction is the adherens junction.

Tight junctions are formed by a series of transmembrane proteins. The first to be described was occludin. Occludin is a 60-65 kDa proteins that span the membrane four times with a long carboxy-terminal cytoplasmic domain and a short amino-terminal cytoplasmic domain. The carboxy-terminal interacts with the cytosolic face of the junctional scaffolding proteins of the *zonula occludens* (ZO) family. Experimental data suggest whilst occludin plays an important role in both barrier and boundary functions, it is not the only molecule involved because selective removal of occludin or use of a mutant occludin does not completely abolish barrier function (Lapierre 2000) (**Fig 1.12**)

Perhaps more importantly than the occludins are the family of claudin proteins. Claudins are integral components of the tight junction strands and also possess four transmembrane domains. Many members of the claudin family have been identified in humans and mice with growing evidence that these proteins constitute the backbone of the tight-junction strands with certain members of the family expressed in certain cells e.g. claudin-5 is expressed primarily in the endothelial cells of blood vessels (Morita *et al.* 1999). The claudin carboxy terminal is able to interact with ZO proteins (Itoh *et al.* 1999).

The last transmembrane protein to be identified within the tight junction is a 40 kDa protein called junctional adhesion molecule (JAM), of which there are three types JAM A, B, C. It is structurally different from the previous two proteins because it belongs to the Immunoglobin (Ig) superfamily and contains a single transmembrane domain.

A series of scaffolding proteins are localized to an area just cytoplasmic to the tight junction. These are ZO-1, ZO-2 and ZO-3 and belong to the MAGUK family of proteins (Membrane Associated Guanine Kinases). The MAGUK proteins are a group of cytoplasmic proteins, which lie close to the membrane and are involved in organizing the structural and functional linkage between transmembrane proteins, the cytoskeleton and signalling pathways.

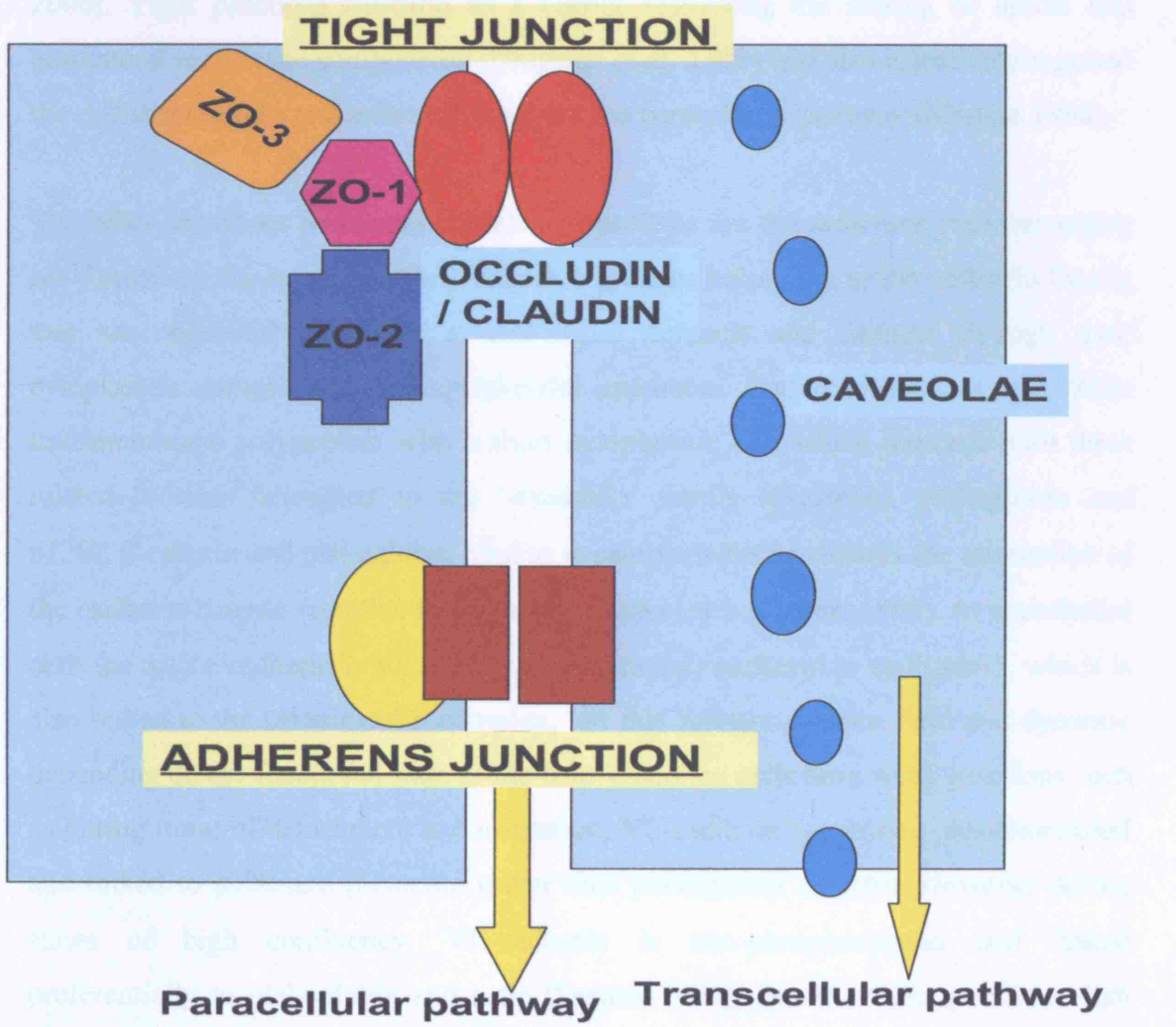


Figure 1.12 Schematic Diagram of Paracellular and Transcellular Pathways

ZO-1 is a 220 KDa protein with a number of multiple predicted phosphorylation sites (for both tyrosine and serine / threonine phosphorylation) which, like occludin, is thought to regulate junctional function (Antonetti *et al.* 1999; Singer *et al.* 1994) (Tsukamoto and Nigam 1999). ZO-1 interacts with occludin and with other tight junction localized proteins including ZO-2, ZO-3, AF-6 and actin.

Actin, which forms part of the cytoskeleton, circumscribes the cell at the level of the adherens junction with fine filaments of actin extending into the tight junction (Hirokawa and Tilney 1982). Regulation of the actin cytoskeleton by GTPases can influence paracellular permeability (Hall 1998). Actin interacts with ZO-1 (Lapierre 2000). Tight junctions function as a barrier restricting the mixing of apical and basolateral membrane components (Dragsten *et al.* 1981) and also selectively regulate the diffusion of ions and solutes throughout the paracellular pathway (Madara 1998).

The other important junctional complex of proteins are the *adherens proteins* which are formed by the transmembrane adhesive proteins belonging to the cadherin family that are organised to cluster at cell-to-cell contacts and connect through their cytoplasmic domain with the cytoskeletal apparatus. Cadherins are a single chain transmembrane polypeptide with a short cytoplasmic tail, which interacts with three related proteins belonging to the 'armadillo' family (β-catenin, plakoglobin and p120). β-catenin and plakoglobin bind to α-catenin, which mediates the interaction of the cadherin-catenin complex to the actin cytoskeleton (Dejana 1996). In endothelial cells the major cadherin is VE (vascular endothelial)-cadherin or cadherin-5, which is also linked to the catenin-actin complex, but this linkage is more fluid and dynamic depending on the functional state of the cell. When the cells have weak junctions such as during times of detachment and migration, VE-cadherin is tyrosine phosphorylated and linked to p120 and β-catenin rather than plakoglobin or actin. However during states of high confluency, VE-cadherin is non-phosphorylated and linked preferentially to plakoglobin and actin (Dejana 1996). Based on this profile switch and *in vitro* work, the VE-cadherin –catenin complex is also important in the control of paracellular permeability (Navarro *et al.* 1995) (**Fig 1.12**).

The tight junctions between the endothelial cells create a selective barrier to water and solutes blocking flux across the tissue and preventing lipids and proteins from diffusing between the apical and basolateral plasma membranes. The influence of neighbouring astrocytes and Muller cells and a contribution of adherens junction may also play a role in preventing the free flow of water and proteins into the interstitial space. There is growing evidence that these neighbouring cells are able to secrete factors which help to maintain the integrity of the endothelial barrier (Gardner *et al.* 2002). Given this ability of glial cells to induce and strengthen barrier properties, and the anatomical arrangement which means that there are no Muller cells present in the central macula and only a few astrocytes present at the edge of the fovea, it is possible to provide an anatomical explanation for the development of oedema in the macula compared to other regions of the retina (Gardner *et al.* 2002).

1.2.4. Oedema

The term 'oedema' describes swelling from fluid but does not specify where the fluid resides. It may develop as a result of water accumulation in the interstitial spaces (described as extracellular or vasogenic oedema, characterized by cell compression) or within cells (intracellular or cytotoxic oedema, characterized by cell swelling). Extracellular oedema is usually associated with excess flux of water from the blood vessels into retinal tissue. Such a flux can occur either by increased hydrostatic forces or by the breakdown of the blood retinal barrier. This can occur either through an increased *transcellular pathway* where the cell junctions remain intact and flux occurs through the cell or damage or disturbance of the tight junction called the *paracellular pathway* with the flux occurring between the cells (**Fig 1.12**).

Oedema is triggered by acute events such as hypoxia, ischaemia or inflammation and maybe aggravated by chronic conditions causing a general weakening or impairment of the BRB including diabetes, hypertension or other vascular disorders. Inflammatory mediators e.g. prostaglandins and VEGF have been suggested to mediate a breakdown of the BRB. The source of these mediators could be either from the leukocytes adhering to the vascular endothelium or from tissue resident cells such as microglial cells, Muller cells and RPE. The increased permeability may occur at either the inner or outer barriers.

Once water has entered the retinal interstitium, it accumulates either in the inner nuclear layer of the inner retina and the photoreceptor inner connecting fibre layer or Henle's layer in the outer retina (or outer plexiform layer). This location seems to be determined by the presence of two high resistance barriers against fluid movement, the inner and outer plexiform layers and by the physical constraints imposed by the organization of the retina.

Under normal physiological conditions fluid can move across the retina and through the barriers. There is evidence that diffusion limits of solutes dissolved in the aqueous phase for metabolic supply in the system are approximately 150 μm. If solutes were leaking from the inner retinal vessels at an abnormal rate and pooling at this diffusion limit, it would change the microanatomy. Increasing pressure within this pool would lead to increasing compactness in the surrounding tissues, displacement of nuclei in the INL and ultimately compression of fibres within both plexiform layers. Fluid could then move into the photoreceptor inner segments and nuclei before compressing junctions in the OLM and enhancing its barrier properties. In conditions in which the outer BRB leaked, fluid would rapidly reach the photoreceptor inner segments and outer aspect of the OLM. It could then pass through this barrier before become further impeded by the OPL. Therefore fluid leaking from the retinal capillaries in the INL would be relatively constrained by the OPL, whereas fluid from the RPE would also be constrained by the OPL The barrier properties from the two plexiform layers probably results from the fact that the extracellular space is highly convoluted and extremely narrow (Reichenbach *et al.* 1988).

As mentioned above, it has also been suggested that intracellular swelling (oedema) of glial cells may significantly contribute to the development of macular oedema with cysts being formed by swollen and dying Muller cells (Fine and Brucker 1981) with some suggesting that swelling of Muller cells may precede extracellular oedema formation (Yanoff *et al.* 1984). This view can be compatible to clinical observation. Increased vascular leakage cannot always be correlated with retinal tissue swelling and functional impairment and not always correlate with angiographic signs of vascular leakage (Marmor 1999).

Several mechanisms have been proposed for intracellular swelling, all centred on an increased drive for water accumulation within the cell. Endocytosis of serum proteins which forces intracellular water accumulation (Bellhorn 1984), increased influx of Na+ ions coupled with elevated glial glutamate uptake secondary to injury-induced neuronal glutamate release would produce increased osmotic pressures for water accumulation (Izumi *et al.* 1999) and lastly a disturbed osmolyte redistribution at the blood-glial cell interface may favour cell swelling (Pannicke *et al.* 2004).

1.2.5. Blood-Retinal Barrier Leakage (Vascular Permeability) in Diabetes

One of the principal mechanisms suggested for the development of macular oedema is the breakdown of the blood retinal barrier (BRB). Although each retinal compartment as described above may show increased permeability in diabetes, the retinal vasculature is the predominant site of leakage in early experimental diabetes (Lobo *et al.* 1999) and human diabetic retinopathy (Vinores *et al.* 1990).

In the rat diabetic model, the retinal vasculature becomes permeable to sodium fluorescein within 8 days after the onset of diabetes (Do Carmo *et al.* 1998) and to albumin after 3 months (Antonetti *et al.* 1998). Vascular endothelial growth factor (VEGF) can increase vascular permeability and is also increased in diabetes (Amin *et al.* 1997) (Aiello *et al.* 1994) (Murata *et al.* 1995).

However using HRP (Horseradish Peroxidase) as a marker of BRB integrity, leakage was demonstrated at the level of RPE but not through the retinal vasculature in streptozotocin-induced (Tso 1982) or in spontaneously diabetic rats (Blair *et al.* 1984). Alternatively other investigators have found HRP leakage from the retinal vasculature (Ishibashi *et al.* 1980), or even found HRP permeability from the RPE in control rats (Kirber *et al.* 1980). HRP has been criticised as a tracer because it can itself induce increased vascular permeability (Cotran and Karnovsky 1967), may be cytotoxic (Balin *et al.* 1986), and has a relatively high molecular weight that may limit its sensitivity as a tracer.

The use of foreign substances as tracers has attracted criticism because of the possible effects of the tracer on the host cell. Therefore the use of endogenous markers as an

alternative system to localise BRB leakage site has gained wide acceptance. Using immunolocalisation for extravascular albumin in spontaneous diabetic and galactosaeimc rats, albumin staining was seen on the abluminal side of retinal capillary endothelium of the inner retina and the retinal side of the RPE. According to the study no disruptions of tight junctions were seen, however there seemed no direct evaluation of tight junction structure to assess this. Applying the technique of evaluating the distribution of extravascular albumin in diabetic post-mortem specimens, the breakdown of the BRB was localised to the inner retina (Vinores *et al.* 1990) and in the rabbit model the opening of the tight junctions was seen but not necessarily in diabetic humans, rats or galatosaemic rats (Vinores *et al.* 1998). In a another study involving diabetic rabbits the results suggested there was increased vesicular transport combined with leakage through the inter-endothelial cell spaces occurring when adjacent endothelial cells showed degenerative changes (Vinores *et al.* 1998). Yet in the alloxan diabetic dog model with retinopathy, abnormal passage of HRP occurred through disrupted tight endothelial cell junctions (Wallow and Engerman 1977).

Therefore these conflicting experimental data suggests that in diabetic retinopathy the increased BRB breakdown can occur by the opening of tight junctions between retinal vascular endothelial cells or RPE cells, by an upregulation of trans-endothelial vesicular transport or by increased surface membrane permeability of RVE or RPE cells resulting form degenerative changes associated with the disease process (Vinores *et al.* 1990).

The vesicles, which have been suggested to contribute to the part of the increased trans-endothelial transport, are thought to be a variant of the vesiculovacuolar organelle (VVO). VVOs were first described in the venular endothelium at sites of enhanced permeability of macromolecules induced by VEGF-A (Dvorak *et al.* 1995), in venules associated with experimental tumours (Dvorak *et al.* 1995) and in animal models of allergic eye disease (Dvorak *et al.* 1994).

VVO aggregate to form grape-like clusters located primarily near the lateral border of endothelial cell allowing for direct communication between the vascular lumen and ablumen. The individual vesicles and vacuoles comprising VVOs are bounded by

membranes and have proteins called caveolins, interconnected by stomata that are closed by thin diaphragms (Feng *et al.* 1996). VVOs provide a continuous pathway across the venular endothelium extending from lumen to ablumen and into the interendothelial cleft. These vesicles and vacuoles of the VVO formation usually seen in non-ocular tissue differ from the caveolae found in capillary endothelial cells in size (being larger) and in organising into a cohesive organelle that occupies up to a fifth of the venular endothelium cytoplasm. The stomata are thought to be regulated by VEGF-A via VEGF receptor 2 (VEGFR-2) which can also be found in the VVO membrane and the luminal and abluminal membranes of the venular endothelium and not to the lateral plasma membranes at interendothelial cell junctions(Feng *et al.* 1999). However in the capillary endothelium this complex structure is not seen, but just shuttling of individual vesicles called caveolae which have a similar membrane structure and caveolin protein within the membrane but are single and do not aggregate to form the larger complex structure of a VVO.

The main mechanism, which has been suggested in mediating this breakdown of the BRB, is the elaboration of soluble factors. A number of soluble factors have been implicated particularly VEGF. Intravitreal injection of VEGF showed opening of the tight junction within 6 hours of the injection with also increase in pinocytic vesicles in RVE cells suggesting active vesicular transport (Luna *et al.* 1997). In a diabetic rat model, the breakdown of the BRB coincided with increased retinal VEGF levels and using fluorescent microspheres localization of the BRB breakdown was attributed to retinal capillaries and venules of the superficial inner retinal vasculature. However the RPE layer was not analysed in this study (Qaum *et al.* 2001).

The likely situation could be one where both the transcellular pathway allowing for the passage of proteins and the paracellular pathway allowing for the passage of water and solutes into the interstitial space exist in the diabetic retina.

CHAPTER 1.3 HYPOTHESIS AND AIMS

The broad aim of the thesis is to investigate the hypothesis that the pathogenesis of diabetic macular oedema involves the following mechanisms:

1. Vitreomacular traction
2. Angiogenic growth factors / and other contributing cytokines
3. Inflammatory
4. Haemodynamic

As this would require a major programme of research, I have concentrated on the following questions:

- What is the role of vitreomacular traction in diabetic macular oedema (DMO)? Does pars plana vitrectomy and posterior hyaloid removal with or without internal limiting membrane peel remove the effect of such tractional forces and so reduce or resolve the level of macular oedema?
- What are the ocular fluid levels of permeability growth factors in DMO and how do they correlate with the foveal thickness and macular volume? How do these levels change post-vitrectomy in comparison with structural macular changes?
- What are the concentrations of other contributing cytokines e.g. angiopoietins, angiostatic cytokines e.g. PEDF and inflammatory and haemodynamic cytokines. Does a change in the angiogenic / angiostatic balance contributes to the development of diabetic retinopathy and diabetic macular oedema?
- To evaluate if effects of cytokines on junctional complexes in a retinal endothelial immortalized cell line is comparable to that of primary rat retinal endothelial cells.

CHAPTER 2

METHODS AND MATERIALS

CHAPTER 2.1. CLINICAL METHODS

All patients were recruited in accordance with the Declaration of Helsinki and with the approval of the Ethics Committee of Moorfields Eye Hospital.

Study patients

Patients attending the Medical Retina clinics of Moorfields Eye Hospital who were diagnosed with diabetes mellitus as per the criteria of the *American Diabetic Association and* with persistent clinically significant macular oedema (CSMO) despite conventional ETDRS macular photocoagulation were offered recruitment into the two principal studies:

1. Prospective observational study of pars plana vitrectomy (PPV) (Study 1)
   Study 1a) without internal limiting peel (ILM) and
   Study 1b) with ILM peel
2. Randomised controlled trial of PPV vs. standard ETDRS macular laser photocoagulation (Study 2).

Patients underwent

- Best-corrected visual acuity (BCVA) using an ETDRS vision score by a masked observer
- Dilated fundoscopy
- Fundus fluorescein angiography (FFA),
- Optical Coherence Tomography (OCT) and
- Fine Matrix Mapping.

These examinations were performed

- Up to and no more than two weeks prior to surgery (baseline) and
- 2, 6, 12, 24 and 48 weeks in the post-operative period.

Inclusion criteria included

i) persistent CSMO involving the foveal centre for < 2 years
ii) previous treatment with macular laser and
iii) ETDRS vision score of 59 to 35 (equivalent Snellen visual acuity between 20/64 and 20/200).

Exclusion criteria were

i) posterior vitreous detachment diagnosed by the presence of a Weiss ring
ii) macular traction diagnosed on fundoscopy and
iii) macular ischaemia as defined by an enlarged foveolar avascular zone (FAZ >1000μm in any dimension and the size judged in relation to the disc diameter) or significant perifoveal capillary loss (which was defined as either loss of capillary network or of increased intercapillary spacing affecting 180^{o} of the perifoveal capillary bed). These parameters were assessed on the arteriovenous phase of the intravenous fundus fluorescein angiography (FFA).
iv) co-existent ocular disease

All patients underwent clinical examination including dilated fundoscopy, undilated best corrected ETDRS and Snellen visual acuty by a masked observer, FFA by the photographic departement at Moorfields Eye Hospital, Optical Coherence Tomography (OCT) and Fine Matrix Mapping (FMM).

FFA was performed after due consent and intravenous fluoroscein (5 mls at 20% fluoroscein concentration) was injected and the first pass of the dye recorded and then continued recording up to 5 minutes post injection. The same camera and hence magnification factor was used to ensure consistency of images for assessment especially of the FAZ.

OCT is a non-invasive, non-contact imaging modality producing high-resolution cross-sectional tomographs of ocular tissue. It produces a 2-dimensional false colour image of the back-scattered light from different layers of the retina analogous to

ultrasonic B-scan imaging. The only difference is that optical reflectivity is measured. Axial resolution up to 14 μm is achievable. Six 6 mm diameter radial scans centred on the point of fixation (where an internal fixation marker is used for the patients to maintain fixation and provide reliability of fixation) are taken at each time point, and assessed qualitatively and analysed quantitatively using the retinal mapping software (Zeiss-Humphrey, Dublin, CA, software version A5). OCT was employed to give 1) macular profile (single horizontal scan through the foveal centre), 2) foveal thickness and 3) macular volume calculated from 6 radial scans centred on the fixation and averaged using quantitative assessment software. This software has a reproducibility level of ± 6% in measuring retinal thickness in diabetic patients (Massin *et al.* 2001). As both the foveal thickness and macular volumes (*the structural indices*) were analysed by the internal software then measurement and / or observer bias from manual calculation are negligent even though I performed the test myself. This was the OCT 2 model and was the only one employed during the course of all the trials.

Fine Matrix Mapping is a psychophysical method of examination, which determines the cone thresholds in a 9 by 9 degree field centred on the point of fixation. 100 points within this test field are analysed using a modified Humphrey field analyser. A 3-dimensional representation of photopic white stimulus thresholds (given as log units) can be generated from this data. The lower the threshold, the better the cone function. A masked observer also performed this test.

Patient recruitment for the three prinicpal trials was complete within 18 months of the start. In total 42 patients were recruited for the three trials but overall 80 patients were referred for consideration in the trials. All patients who were offered entry into the trials accepted participation.

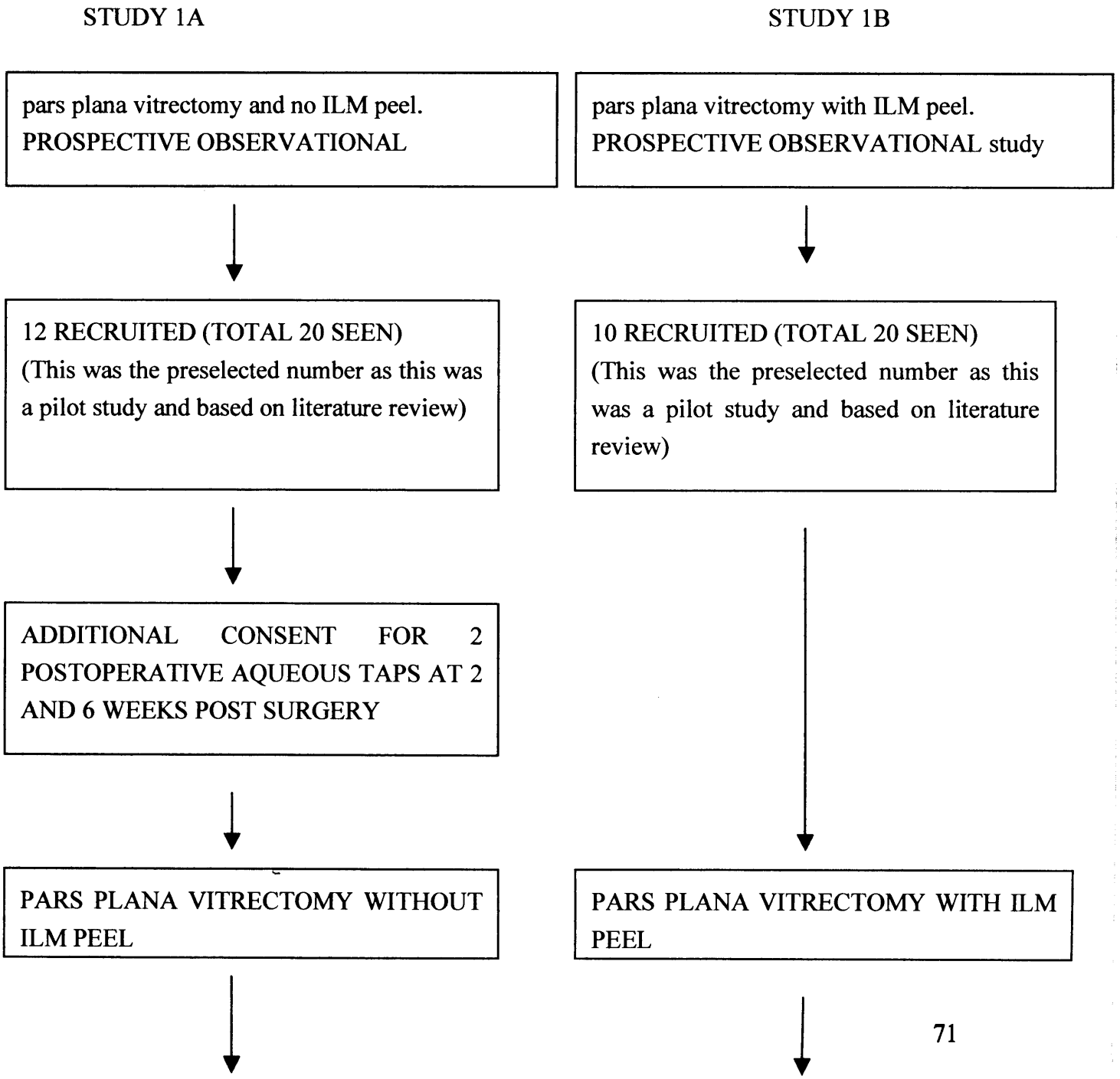
Surgical Technique

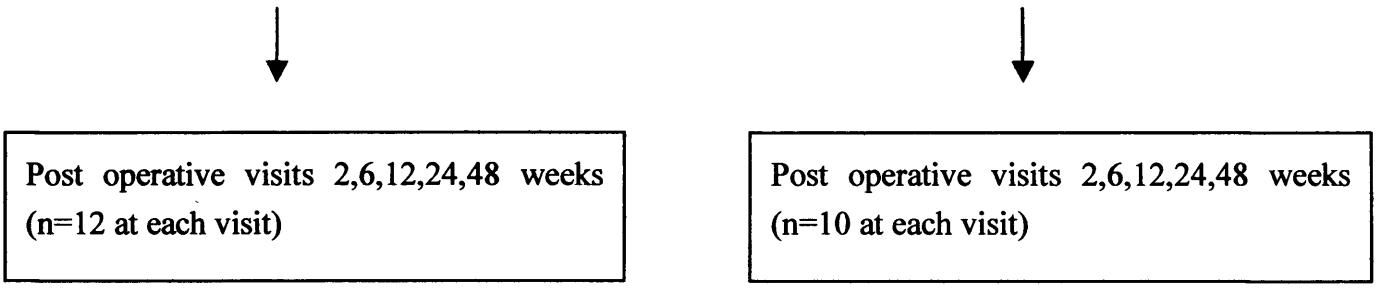
Patients undergoing PPV underwent standard 3-port PPV with elimination and removal of the posterior vitreous cortex with one cohort having no ILM peel and another cohort having an additional peel of the internal limiting membrane. Fluid-sulphur hexafluoride (SF_6) gas exchange was performed if retinal breaks were found

on the 360° examination of the peripheral retina prior to the conclusion of the operation. Such breaks were treated with laser photocoagulation or cryotherapy. Subconjunctival injection of beclamethasone and cefuroxime was given at the conclusion of the operation. Patients were treated with topical Atropine 1%, dexamethasone and chloramphenicol for 3 weeks after the operation. Patients undergoing macular laser underwent standard ETDRS macular photocoagulation.

Study 1a and 1b

Patients were recruited as per the criteria and underwent the clinical studies at the time points described above.

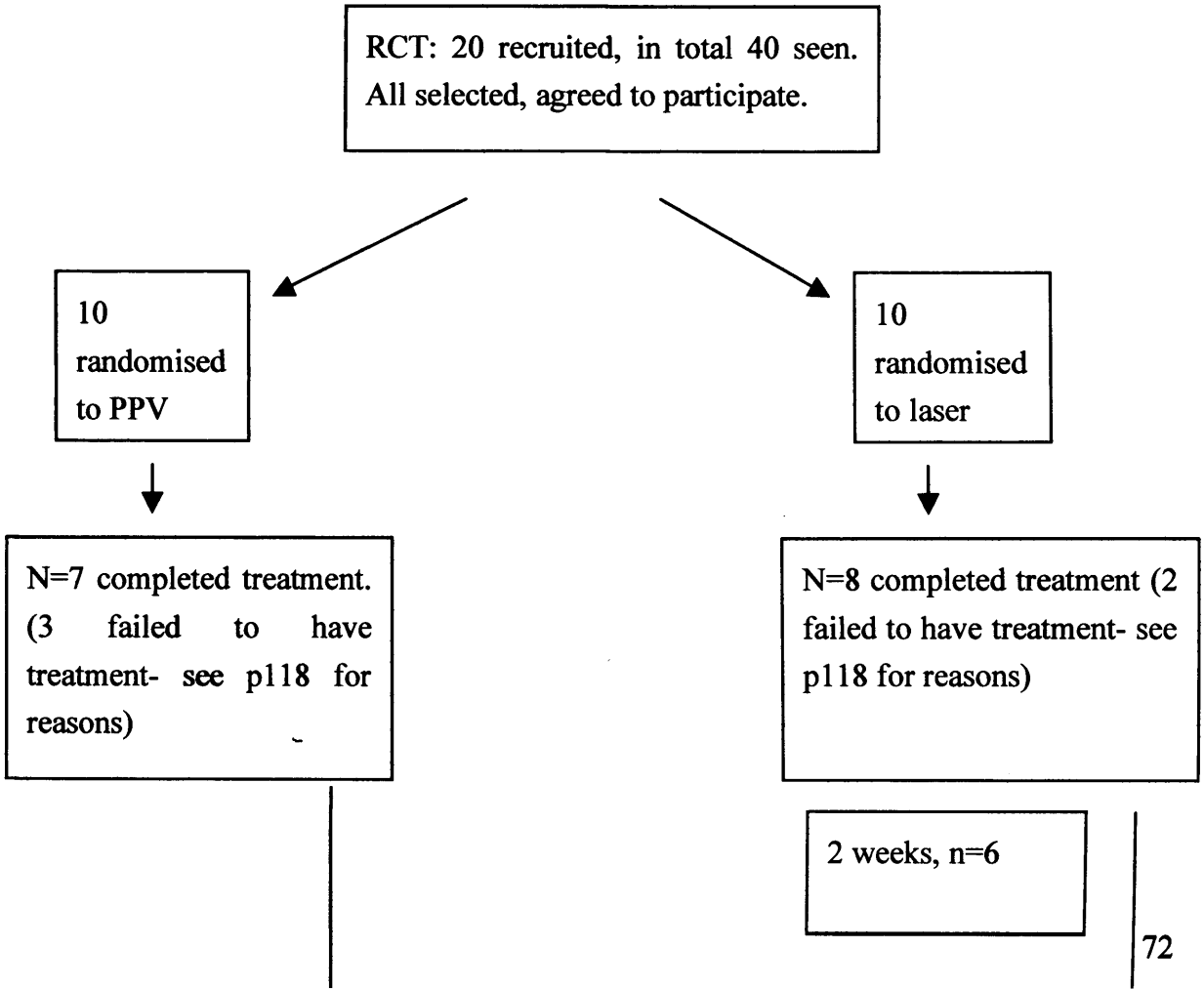


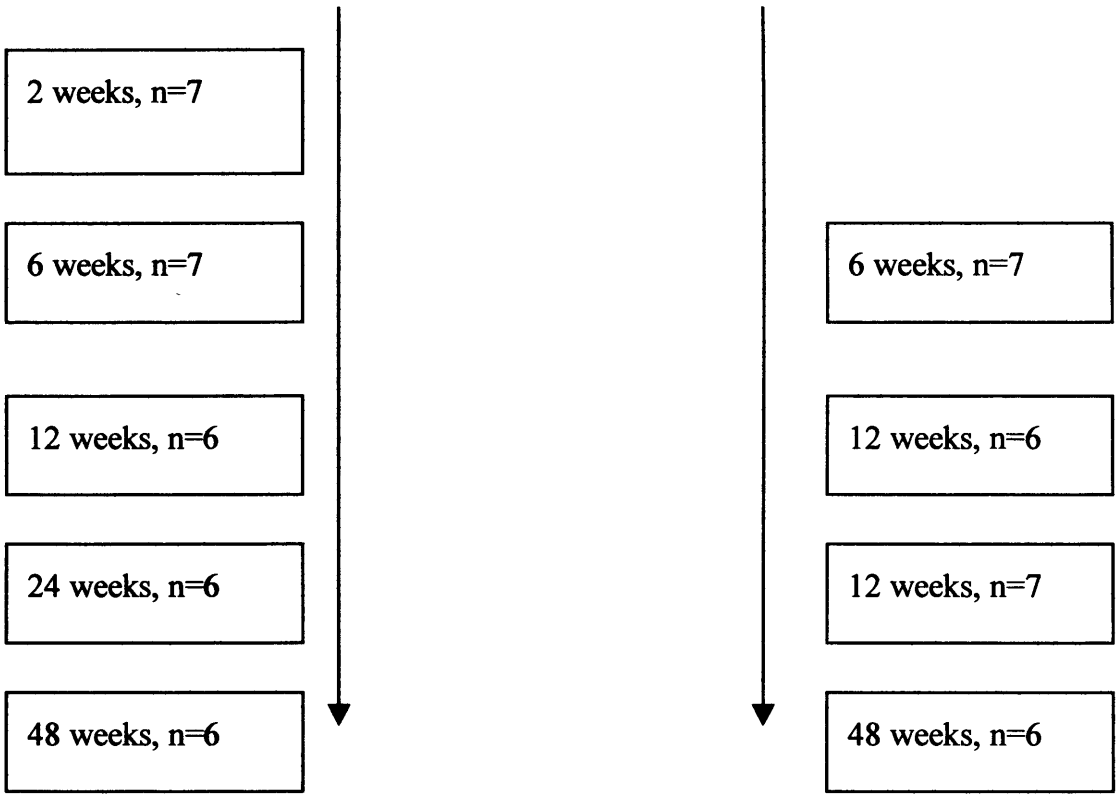


Study 2

This was a pilot randomised control trial (RCT) to determine the number needed in the definitive RCT of PPV without ILM peel against standard ETDRS argon macular laser. Twnety patients in total were recruited and randomised 1:1 between the two treatment modalities.

PPV was performed as described above. For those randomised to laser, the baseline FFA was used to determine the location and extent of laser treatment needed as per ETDRS using an Area Centralis Volk Lens ™ at spot size 100 μm, duration 0.08 secs, and a power starting at 80 mw but increasing to achieve a soft grey retinal reaction.





At each visit each patient attending follow up assessment from either treatment arm underwent full examination protocol.

Reference Groups

Vitreous samples from two groups of control patients were used as comparison to the study patients.

Patients with idiopathic full thickness macular hole (FTMH) and patients who had been previously lasered with panretinal photocoagulation for proliferative diabetic retinopathy (PDR) and were undergoing PPV for complications related to the PDR. In these patients neovascularization was considered active if the patient had perfused multibranched preretinal capillaries or quiescent if the patient had gliotic nonperfused vessels and / or tractional retinal detachment associated with inactive fibrovascular membranes but not involving the macula. These PDR patients had been previously lasered as part of their treatment but were listed for PPV for the indications listed above. Hence from the PDR group, vitreous from either active or quiescent proliferative disease was used to compare the concentration of cytokines found in these patients against the vitreous from the diabetic macular oedema patients.

Statistical Analysis

Clinical parameters and OCT were analysed by unpaired Mann Whitney U test and a paired Wilcoxon test used for the appropriate comparison and the significance value taken at $p<0.05$. Nonparametric tests were used due to the small numbers of patients used in each of the clinical studies especially in the RCT which was a pilot study performed to ascertain the power for a full RCT study.

CHAPTER 2.2 LABORATORY METHODS

2.2.1 Chemiluminescence

Immunoassays using labelled reagents for detecting antigens are extremely sensitive and economical in the use of reagents. There are many forms of immunoassays. The most widely used type are solid phase assays for antibodies employing ligands labelled with enzymes or radioisotopes. Recently chemiluminescent markers are tending to replace radioisotopes for labelling.

The main advantage of chemiluminescence (CL) is the high detectability of the luminescence signal which can be measured down to a few emitted photons in a luminometer ie CL can achieve detection limits down to a few molecules or 10^{-18} to 10^{-21} mol (Roda *et al.* 2004).

There were two main type of immunoassays used in this study. They were the Two-site Capture Assay and the Competitive Assay.

The two-site capture (or sandwich) assay (**see Figure 2.1 a**) involves coating the assay plate with the primary antibody. The test solution is added and the antigen is then bound to the primary antibody. After washing off unbound antigen, the captured antigen is then detected using another antibody, which binds another epitope of the antigen. This detection antibody is usually labelled: in this study it was biotinylated.

Given that in this assay two different antibodies detect the antigen, the second in excess, makes this assay both highly specific and sensitive and combined with the chemiluminescence detection system, then the minimum detection concentration of the antigen can be very small. Most of the assays performed were of this type.

The competitive assay involved pre-coating the test plate with antigen and then adding a solution which contains either a standard concentration of test antigen or the test solution which has an unknown concentration of the antigen together with known concentration of antibody specific to the antigen. If there is excess antigen in solution then there will be limited antibody reaction with the precoated antigen and therefore after a secondary labelled antibody is added and developed, a minimal signal is seen using the luminometer (**see Figure 2.1 b**), *vice versa* if minimal antigen is present in the standard test or unknown sample solution (**see Figure 2.1 b**). This is a negative competitive assay, used here to determine the PEDF concentration in ocular fluids.

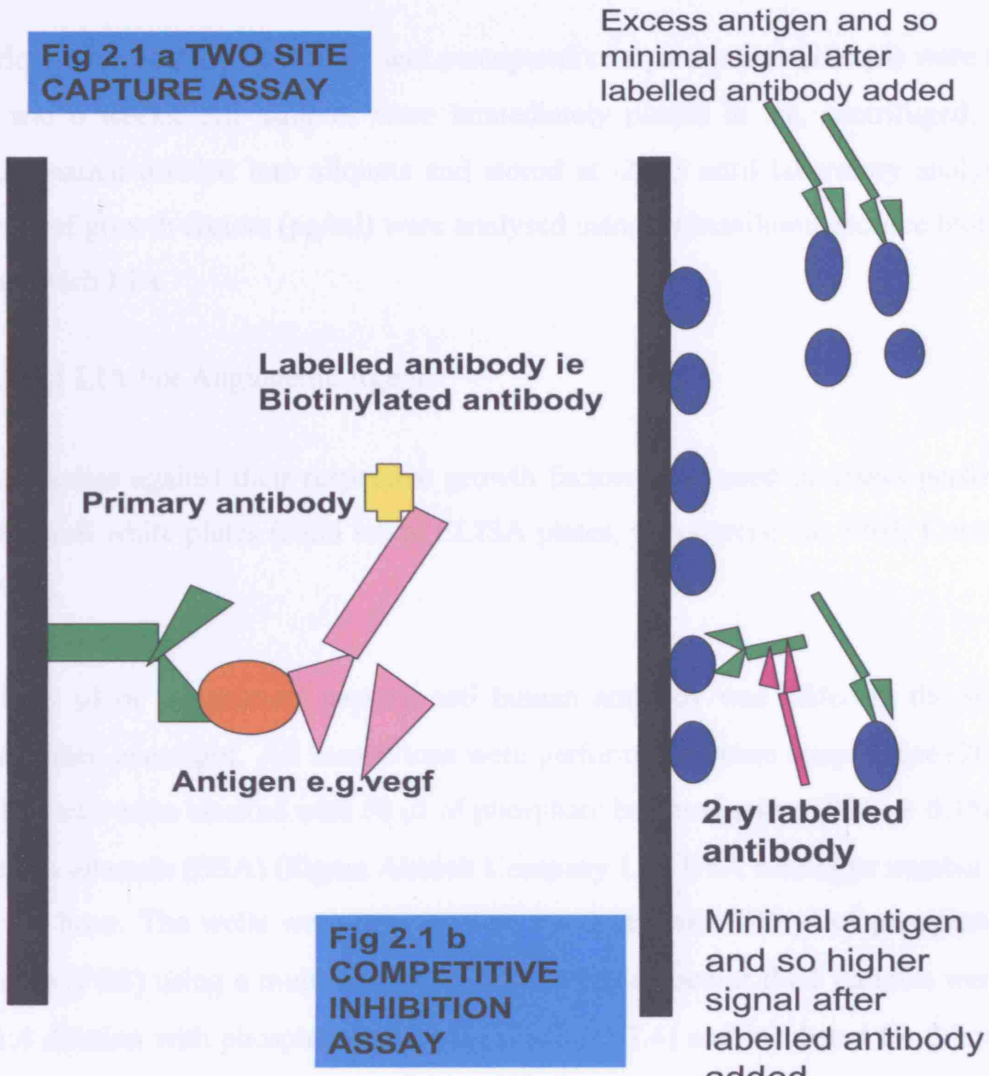


Fig 2.1

Figure 2.1 Two Types of Immunoassays: Two Site Capture and Competitive Inhibition Assays.

2.2.2 Luminescent Immunoassay (LIA) (see Table 2.1 for sensitivities of the and coefficient of variation –CoVs- for each assay)

Evaluation of the levels of growth factors and cytokines in the ocular fluids was performed by LIA.

Baseline vitreous (up 500 μl) and aqueous (100 μl) samples were taken immediately

prior to the start of vitrectomy and postoperative aqueous taps (100 μl) were taken at 2 and 6 weeks. All samples were immediately placed in ice, centrifuged, and the supernatant divided into aliquots and stored at -20°C until laboratory analysis. The level of growth factors (pg/ml) were analysed using a chemiluminescence biotinylated sandwich LIA.

2.2.2.1 LIA For Angiogenic Agents

Antibodies against their respective growth factors were used in assays performed in 384-well white plates (solid white ELISA plates, polystyrene No 3705, Corning BV, USA).

Thirty μl of the primary capture anti human antibody was added to the wells and incubated overnight. All incubations were performed at room temperature (20-25 °C). The wells were blocked with 50 μl of phosphate buffered saline (PBS) + 0.1% bovine serum albumin (BSA) (Sigma Aldrich Company Ltd, USA catalogue number A9647) for 1 hour. The wells were then washed six times with 100 μl of phosphate buffer saline (PBS) using a multi-channel pipette. 30 μl of ocular fluid samples were added (1:4 dilution with phosphate buffered saline, pH 7.4) and incubated for 2 hours. The wells were then washed four times (50 μl) with PBS. Thereafter, 30 μl of the human biotinylated affinity purified detection or secondary antibody followed by the addition of 30 μl of Neutravidin ™ Horseradish Peroxidase Conjugated (Pierce, Illinois, USA) at 0.125 μg/ml, each for 30 min. Neutravidin ™ can bind four biotins per molecule with high affinity and selectivity. Before the addition of Neutravidin, a repeat washing (four times) of the wells was performed with PBS (50 μl). Finally 30 μl of LumiGLO® Reagent (a chemiluminescent luminol-based peroxidase substrate from New England Biolabs, USA) and Peroxide was added for 5 minutes and the plate read in a luminometer. Phosphate Buffered Saline (PBS) was used for washing the wells, with the final wash, the one before the addition of LumiGLO, being Tris- Buffered Saline (TBS 50mM pH 8) (Sigma Aldrich Company Ltd, USA, catalogue number T-5030), using a multipipette channel. All sample readings were quantified from a standard curve produced using recombinant human cytokine (from R & D).

The nature and the concentrations of each pair of antibody for each growth factor and their respective inter- and intra-assay coefficient of variation (CoV) and concentration sensitivity are shown in Table 1. All antibody pairs and standard growth factor peptides were obtained from R and D Systems, Minneapolis MN, USA. Therefore the concentrations of the cytokines and growth factors mentioned in the table below were determined using the antibody pairs at the concentrations given as part of a two-site (or sandwich) assay.

Table 2.1: Antibody profile for LIA tests

	Primary antibody concentration (ng/ml)	Secondary capture concentration (ng/ml)	Inter Assay CoV	Intra Assay CoV	Sensitivity
VEGF-A (isoform 165)	Polyclonal 125 ng/ml	Polyclonal, biotinylated 100 ng/ml	10%	3%	1 pg/ml
HGF	Monoclonal, 2000 ng/ml, Clone No= 24516	Biotinylated, 250 ng/nl	14%	1%	5 pg/ml
TGF β1	Monoclonal, 2000 ng/ml, Clone No= 9016	Biotinylated, polyclonal 100 ng/ml	16%	7%	1 pg/ml
MMP 9	Monoclonal, 2000 ng/ml, Clone No= 4H3	Biotinylated, polyclonal 100 ng/ml	14%	12 %	1 pg/ml
IL-1 β	Polyclonal, 2000 ng/ml	Biotinylated, polyclonal 200 ng/ml	17%	18%	1 pg/ml
IL-1 Ra	Monoclonal, 2000 ng/ml, Clone No= 10309	Biotinylated, polyclonal, 200 ng/ml	21 %	21 %	0.5 ng/ml

Soluble flt-1 Receptor	Monoclonal 500 ng/ml, Clone No= CG606	Biotinylated, polyclonal 400 ng/ml	20%	7.5%	100 pg/ml

2.2.2.2 Intravitreal Protein Concentration

This was determined using the Bradford assay (Bradford 1976) using bovine serum albumin (BSA) as the standard. BSA standards from 0-25 μg/ml were made from stock aliquot of 10 mg/ml and 5 μl of samples were used. To each concentration of standard and sample 1 ml of a 1:5 diluted Bradford Reagent (Sigma Aldrich Company Ltd, B6916) was added. Then 200 μl of each sample and standard was added in duplicate to a 96 well plate and then read at 570 nm in a colorimeter. The sample protein concentration was determined in relation to the standards produced.

2.2.2.3 LIA For Angiopoietins.

30 μl FcTie-2 Receptor (Regeneron Pharmaceuticals Inc, USA) was added to wells of a 384-well ELISA plate and incubated overnight at 4^0C at a concentration of 3 μg/ml. The wells were then washed 6 times with 100 μl of phosphate buffer saline (PBS) and then blocked with 100 μl of PBS– 0.5% of Bovine Serum-Albumin (BSA) for 1 hour at room temperature (RT). Then either 30 μl either angiopoietin 1 (R and D Systems, Minneapolis MN, USA) or angiopoietin 2 (Regeneron Pharmaceuticals Inc) standards or samples were added to the wells for 2 hours at RT (samples had 1:4 dilution with phosphate buffered saline, pH 7.4). The wells were then washed 6 times with PBS (50 μl), thereafter either 30 μl of NTL-1 (secondary antibody for angiopoietin 1) or NTL-2 (secondary antibody for angiopoietin 2) (both secondary antibodies were kindly supplied by Regeneron Pharmaceutical Inc, USA) was added to each well depending on which cytokine was tested. These antibodies were used at 1:10,000 in PBS-0.5% BSA. The wells were then washed four times with 50 μl of PBS and 30 μl of a tertiary antibody of HRP goat conjugated anti-rabbit at 1:10,000 for 1 hour at RT was then added. Then the wells were washed with TBS (four times, 50μl) followed by 30 μl of LumiGLO ^{R} Reagent and Peroxide (from New England Biolabs, USA) for 5 minutes and the plate read in a luminometer. The angiopoietin 1 standard curve was from 100

ng/ml to 5 pg/ml with a sensitivity of 5 pg/ml and with the results of the samples falling within this curve; similarly for the angiopoietin 2 results the samples were within the standard curve from 100 ng/ml to 1 ng/ml and a sensitivity of 1 ng/ml. Vitreous samples were tested in triplicate and the aqueous samples tested in duplicate.

2.2.2.4 LIA For Pigment Epithelial Derived Factor (PEDF): A competitive inhibition assay

The assay was performed in 384-well plates. 30 µl of 10 µg/ml PEDF were incubated overnight at 4 C. Then PEDF standards or samples were incubated with PEDF antibody at 1:1000 for 1 hr at room temperature (RT). The wells were blocked with 50 µl of Phosphate Buffered Saline (PBS) Bovine Serum Albumin (BSA) 0.5 % for 1 hour at RT. The wells were washed with 100 µl PBS six times. 30 µl of samples and standards in duplicate were added to the wells for 2 hours at RT. The wells were washed as before. Then 30 µl of secondary antibody Horseradish Peroxidase (HRP) goat anti-rabbit antibody at 1: 10,000 for 1 hour at RT was added to each well and then washed with PBS. Lastly 30 µl of LumiGLO ^{R} Reagent and Peroxide (from New England Biolabs, USA) was added for 5 minutes and the plate read in a luminometer. The PEDF antigen and PEDF antibody were kindly donated by Dr Joyce Tombran-Tink (Pharmaceutical Sciences, University of Missouri, Kansas City).

2.2.2.5. ELISA for Nitric Oxide, Prostacyclin and Endothelin-1

The vitreal concentration of these growth factors was determined using ELISA kits purchased for R & D Systems, Minneapolis, MN, USA according to the manufacturer's instructions (Prostacyclin Immunoassay Catalogue Number DE 1700 ; Human Endothelin-1 Immunoassay Catalogue Number QET 00 ; Total Nitric Oxide Assay Catalogue Number DE 1600)

The Nitric Oxide assay determines the total nitric oxide based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. This reaction is then followed by a colorimetric detection of the nitrite as an azo dye product of the Greiss Reaction. The Greiss reaction is a two-step diazotization reaction that produces a chromophoric azo-derivative that absorbs light at 540-570 nm. The samples were tested in duplicate as

were the nitrate standards and averaged. The minimum concentration of nitrite detectable (as a measure of nitric oxide) in the samples as was 3.12 μmol/l (intra-assay coefficient of variation was 1.2 % - 5.3 %; the inter-assay coefficient of variation was 3.3 % - 7 %).

The Prostacyclin assay relies on determining the concentration of 6-keto-PGF $_{1a}$, which is a non-enzymatic hydration product of prostacyclin ($PG1_2$). It is a competitive assay where the hydration product competes with a fixed amount of alkaline phosphatase-labelled 6-keto-PGF $_{1a}$ for sites on a sheep polyclonal antibody. This antibody in turn binds to donkey anti-sheep antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to determine the bound enzyme activity with the absorbance is read at 405 nm. The intensity of the colour is inversely proportional to the concentration of 6-keto-PGF $_{1a}$, which corresponds to the $PG1_2$ concentration. The concentration range of detection was 7.8 pg/ml to 2000 pg/ml and all samples fell within this range (intra-assay coefficient of variation 2.9 % - 9 %; inter assay coefficient of variation 3.4 % - 9.4 %).

The Endothelin-1 assay was a quantitative sandwich enzyme immunoassay. A monoclonal antibody specific for endothelin-1 (ET-1) is precoated onto the microplate. Standards and samples are added and any ET-1 becomes bound to the antibody, which is then detected by adding an enzyme-linked monoclonal antibody to ET-1, which after further washing is determined using a luminol-peroxide substrate and a luminometer to measure the intensity of the light emitted. The concentration range of detection was 0.32 pg/ml to1000 pg/ml and all samples fell within this range (intra-assay coefficient of variation 1.5 % - 2.5 %; inter assay coefficient of variation 5.4 % - 10 %).

All assays were run in triplicates and the intraobserver CoV was calculated by perofming the assay on the sample sample three times at the same time and the interobserver CoV was calculated from performing the assay on the same sample on three different occasions.

Statistical Analysis

Nonparametric analysis was performed to assess for significance given the numbers of samples. The Mann-Whitney U-test was used to analyse the different angiogenic cytokine concentrations and vitreal protein concentrations between the two groups. Growth factor correlations were assessed using the Spearman rank test. Values are reported as medians with ranges and line graphs demonstrate the median changes with ranges given in the text. Two-tailed test results were considered significant at $p<0.05$.

CHAPTER 2.3. CELL CULTURE METHODOLOGY

2.3.1 Cell Culture Protocol for Immortalised Rat Retinal Endothelial Cells (JG2)

Collagen coating of cell culture flasks (T75, NUNC) was carried out using type I collagen from calf skin (C-8919, Sigma Aldrich Company Ltd, USA) which was diluted at a concentration of 1: 20 using Hank's Balanced Salt Solution (HBSS) (Gibco BRL, Invitrogen Ltd, UK). After 1 hour this solution was removed and the collagen was then fixed using an ammonia hydroxide chamber for 10 to 20 minutes. The collagen was then neutralized with further HBSS before culture media was added.

Culture media consisted of 400 ml of F-10 HAM with L-Glutamine (Gibco BRL Invitrogen Ltd, UK, 31550-023) with 100 ml Heat Inactivated Fetal Calf Serum (FCS) (Gibco BRL Invitrogen Ltd, UK) (which is equivalent to 20 % FCS) and Penicillin and Streptomycin antibiotic mixture at a final concentration of 100 μg/ml and 100 U/ml respectively (Gibco BRL Invitrogen Ltd, UK).

Stored cells, which were frozen in liquid nitrogen, were initially defrosted in a 37 ^{0}C water bath, added to 10 ml of culture media, and then centrifuged for 5 minutes at 360 *g* (Sanyo MSE, model Harrier 18/80) to form a pellet of cells. The media supernatant was removed and the pellet was resuspended in 5 ml of culture media and then this was added to a cell culture flask prepared as described above. The cells are incubated at 37 ^{0}C with 5 % CO_2. Media was changed every 2-3 days and the cells were grown

until confluence (usually within 1 week), then passaged, and split 1: 3 into new culture flasks.

2.3.2 Cell Culture Method for Primary Rat Retinal Endothelial Cells.

Male Lewis rats were sacrificed in a CO_2 chamber and the eyes and the retina dissected out. The retina was then placed in 10-15 ml of working buffer which contains the following ingredients: 150 ml calcium / magnesium-free Hanks Balanced Salt Solution (Gibco BRL Invitrogen Ltd, UK, 14170-088), 1.5 ml 1M HEPES (Sigma Aldrich Company Ltd, USA H0887) and 1.5 ml Pen / Strep combination (Gibco BRL Invitrogen Ltd, UK, 15070-063) and1.36 ml (of 22 % w/v BSA per 50 ml. It was then centrifuged for 10 minutes at 360 *g*. Then the tissue was digested in 10-15 ml of Digest Medium in a universal (Calcium / Magnesium-free Hanks Balanced Salt Solution containing 20 µl of TLCK at 0.147 µg / ml (Sigma –Aldrich, UK T7254), 1 mg/ml collagenase / diaspase solution (Boehringer Mannheim 269638), 10mM HEPES, 100 i.u. penicillin / 100 µg/ml streptomycin, 2000 units / ml DNAse (Sigma-Aldrich, UK D4263) for 1 hour in the water bath (37°C) and agitated every 15 minutes by tapping on the side of the universal. Then the digested tissue was centrifuged for 10 minutes at 360 *g* and the supernatant was removed and resuspended in 10-15 ml of 22 % (w/v) BSA in PBS and centrifuged again at 3000 rpm for 20 minutes. The supernatant was removed and the remaining pellet re-suspended in 1 ml of working buffer (made of the cocktail of ingredients described above) and transferred to a fresh universal tube, topped up with Working Buffer and centrifuged for 10 minutes at 360 *g*. Again the supernatant was removed and the pellet re-suspended in the remaining Digest medium in the water bath (37°C) for 2 hours. After this it was centrifuged again for 10 minutes at 360 *g*. The pellet was re-suspended in 1-2 ml of working buffer and then this suspended solution was layered onto the Percoll Gradient to perform Density Gradient Centrifugation.

Density Gradient Centrifugation

A 10 ml 'Du Pont Centrifuge' tube was sterilised and filled with 70 % alcohol. This was then decanted off and washed with HBSS three times. The tube was then filled

with Working Buffer and allowed to stand for at least an hour (this pre-coats the tube with protein to prevent microvessels sticking to the wall of the centrifuge tube). About 90 minutes before the end of the second digest incubation period, the buffer is decanted and 7 ml of 50% Percoll solution is added to the tube. The tube is then centrifuged for 1 hour at 4 °C at 4350 *g*. The Percoll solution contains calcium and magnesium in the HBSS in order to aggregate the microvessels.

Once the re-suspended solution had been layered in the Percoll Gradient then the tube is centrifuged for 20 minutes at 900 *g*. The solution should sink below the top of the Percoll if the gradient is properly equilibrated and form capillary fragments (seen as flocculated red particles) after centrifugation in a layer 4/5 of the way down the centrifuge tube. These are removed from this layer with about 2-2.5 ml of the gradient above them. This solution is then re-suspended in working buffer and centrifuged at 450 *g* for 10 minutes. The supernatant is removed and the pellet re-suspended in growth medium / Working Buffer (50:50) and centrifuged again for 5 minutes. The pellet is then re-suspended in the culture medium and plated out on collagen-coated culture flasks and incubated at 37 ^{0}C with 5 % CO_2.

Growth Medium consists of Hams F-10 medium (200 ml), 16 % bovine cell free plasma-derived serum (PDS), 2mM L-glutamine, 100 i.u. penicillin / 100 μg/ml streptomycin, 80 μg/ml heparin (Grade I), 75 μg/ml endothelial cell growth supplement. The Percoll Density Gradient contains 50 ml Percoll, 5 ml 10X HBSS with calcium and magnesium and 45 ml with calcium and magnesium.

2.3.3. Junctional Protein Localisation Following Cytokine Treatment

The cells were washed with sterile HBSS and then allowed to equilibrate in fresh culture media for 2 hours in 37°C under 5% humidified CO_2 before treatment. Cytokine or vitreous from diabetic patients (made up to the required concentration in full equilibrated culture media) was added to the wells for the desired time course. Cytokine concentrations of VEGF (100, 50, 5, 1.5, 0.7 ng/ml), HGF (20, 1.5 ng/ml) and Vitreous from Group 1 and Group 2 was added either for 30 minutes or 24 hours as appropriate. Using the known protein concentration of vitreous from Group 1 and 2, a similar total protein concentration was used.

The cells were then fixed with methanol for 2 minutes at –20 °C. They were then washed three times with sterile PBS and blocked with 10% serum / sterile PBS for 1-2 h at room temperature (RT): for tight junctions, goat serum and for adherens junction, fetal calf serum was used. They were then washed three times with sterile PBS. Primary antibody in 1% serum / PBS was added for 2 h at room temperature: for tight junctions (TJ) rabbit anti zonula occludens -1 (ZO-1) was used. They were then washed 5 times with sterile PBS. Secondary antibodies in PBS were added to the cells for 1 hour at room temperature in the dark: For TJ goat anti-rabbit –FITC (**see table 2.2 for detailed description of antibodies used**). Cells serving as control had only secondary antibody added. All the cells were then washed five times with sterile PBS. They were then blocked with 10% goat serum /PBS for 1 hour at RT and then washed three times with sterile PBS for the next set of primary antibodies.

The second set of primary antibodies used were diluted in 1% goat serum / PBS and added for 2 hours at RT. They were for the Adherens Junction (AJ) protein β-catenin (mouse anti β-catenin). Following addition to the cells they were washed five times with sterile PBS. A secondary antibody for β-catenin AJ was then added in PBS for 1 hour at RT in dark (goat anti-mouse-Cy ^{3}) together with secondary only controls. All the cells were then washed five times with sterile PBS Cells were also used to examine the distribution of caveolin (by the staining of caveolin-1) after similar treatment with cytokines and vitreous together with controls. The primary antibody was a mouse anti-caveolin 1 with a secondary goat anti-mouse Cy ^{3} (see Table 2), which was applied with this panel of antibodies. Also cells for the distribution and effect on actin were stained with phalloidin-rhodamine (see Table 2).

They were then mounted with a cover slip, sealed and stored at 4°C in the dark until viewed by confocal microscopy.

Table 2.2 Antibody Profile for Cell Culture

	PRIMARY ANTIBODY (and dilution used)	**SECONDARY ANTIBODY (And dilution used)**
Zonula Occludens (tight junction)	Rabbit Anti-ZO-1 (1:50)	Goat Anti-Rabbit FITC (1:50)
VE-cadherin (adherens junction)	Goat Anti-VE cadherin (1:200)	Donkey Anti-Goat FITC (1:50)
Caveoiln-1	Mouse Anti-Caveolin-1 (1:500)	Goat Anti-Mouse Cy3 (1:50)
Actin	Phalloidin-Rhodamine (1:50)	None needed

2.3.5 Confocal Microscopy

Standard fluorescence microscopy uses the absorption of higher energy light by a fluorescent molecule, which in turn emits light of a lower energy. In fluorescence microscopy, the microscope has a special dichroic mirror that reflects light of a shorter wavelength and passes light of a longer wavelength. The problem of fluorescence microscopy is that the excitation light illuminates the whole sample. Even though the highest intensity of the excitation light is at the focal point of the lens within the microscope, other parts of the sample also receive this light and also fluoresce which inevitably contributes to the background haze in the resulting image. However if there is a pinhole with a screen at the focal point of the objective lens of the microscope that would then allow light at this focal point to be sharply imaged as it can pass through the pinhole. Light from different wavelengths not imaged at the focal point of the lens would not pass through the pinhole and hence reduce background haze. The pinhole is **con**jugate to the **focal** point of the lens, thus it is a **confocal** pinhole. Therefore confocal microscopy is efficient in rejecting out of focus fluorescent light. Therefore the image comes from a thin section of the sample. By scanning many thin sections from the sample, a three dimensional image of the

sample can be created.

2.3.6 Cell Permeability Assay.

Cells were seeded onto Transwell filters (Catalogue Number 3470, Corning, Acton, MA, USA) and maintained in culture at 37°C under 5% humidified CO_2 until confluence was reached usually within 2-3 weeks, changing the media above and below the filters twice weekly. Filters without cells were also maintained as controls. FITC-dextran molecular weight sizes of 40 and 150 kDa were dissolved in serum free medium to a concentration of 2 mg/ml. To assess the endothelial permeability under the influence of VEGF of the cells in the apical chamber, VEGF was added at a number of different times: 5 and 30 minutes, 1, 2, 4, and 24 hours. Filters with only cells and no cytokine treatment were also used as controls. The different FITC-dextran molecular weights were added after VEGF treatment and removed from the apical chamber. Cell media from the basal chamber was also removed and replaced with 400 µl of serum free media. At 5, 15, 30, 45 and 60 minutes after addition of the media containing FITC-dextran, 50 µl from the basal chamber was removed and diluted in sterile PBS to 1 ml before measurement of fluorescence using a fluorometer at an excitation wavelength of 490 nm and an emission wavelength of 520 nm (Safire, Tecan, Mannedorf, Switzerland). Controls included media from cell free filters, neat FITC-dextran, and cells not treated with VEGF. At each time point, media removed from the basal side of the filter was replaced with fresh serum-free media to maintain volume. The resulting fluorescence reading was taken as per ml and fluorescence reading for each treatment was plotted against time after the addition of the FITC-dextran.

CHAPTER 3

RESULTS

CHAPTER 3.1 CLINICAL STUDIES

3.1.1 Prospective Observational Studies 1a and 1b: Pars Plana Vitrectomy with and without ILM peel for Diabetic Macular Oedema.

Study 1a. PPV without ILM peel

Twelve patients were enrolled in the study (see Table 3.1 for demographics and basic clinical characteristics). All patients had type II diabetes mellitus of an average duration 11 years (range 4-23 years). 4 were on oral medication; 6 were on insulin, only 1 was on combined insulin and oral medication treatment and 1 was controlled on diet alone. The average HbA1c was 8% (range 5.8-9.9). Nine patients had controlled systemic hypertension and one was on lipid lowering drugs. The patients suffered from no other significant ocular conditions. All patients had moderate non-proliferative diabetic retinopathy. All had fovea-involving macular oedema which had been present an average of 15 months (range 12-18 months) and all were previously treated with macular argon laser photocoagulation on average 3 times (range 1-5) prior to surgery.

Per-operative assessment confirmed an attached posterior hyaloid face in all cases and posterior hyaloid separation was induced as part of the operative protocol. There were no pre-operative or post-operative complications resulting from the surgery.

Table 3.1. Basic demographics and clinical characteristics for Study 1a

Number	12
Average age (years)	63
Sex	7 male; 5 female
Diabetic Type	Type 2
Average years diabetes	11
HbA1C (%)	8
Hypertension	9

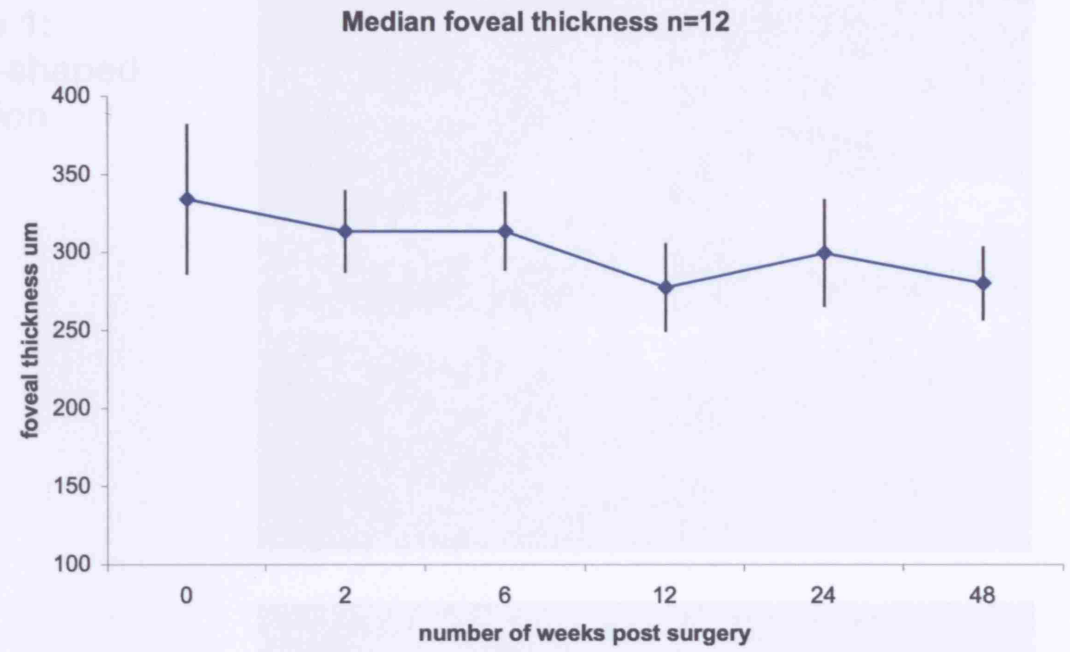
Diabetic Retinopathy	All had moderate non-proliferative diabetic retinopathy
Average number macular laser treatments	3
Average duration of oedema prior to recruitment (months)	15

OCT findings

The foveal thickening for all 12 patients ranged from 183 mm – 751 mm (normal range 126-180 mm) and the macular volume ranged from 2.13 – 6.42 mm^3 (normal < 1.66 mm^3).

After pars plana vitrectomy without peeling of the internal limiting membrane, for all 12 patients, both the median foveal thickness (334 mm at baseline and 280 mm at 12 months) and the median macular volume (3.24 mm^3 at baseline and 2.61 mm^3 at 12 months) showed gradually improvement over the 12 month post-operative period (the difference between baseline and 12 month levels for both parameters was significant, p=0.01) (see Figure 3.1).

There appeared to be two distinct patterns of macular profile on OCT and the patients were divided into 2 groups accordingly. Group 1 (n=4) had a dome-shaped thickened macula with posterior hyaloid partially elevated but adherent to the tip of the fovea and with a large central pocket of fluid within the retina **(see Figure 3.2a)**. Baseline median foveal thickness was 478 μm (range 472-751 μm) and macular volume was 3.9mm^3 (range 3.74-6.42 mm^3). Group 2 (n=8) had a diffuse low-elevation profile of the thickened macula with reduced intraretinal reflectivity and multiple intraretinal **(see Figure 3.2a)** cysts and no signs of a focal posterior hyaloid separation.



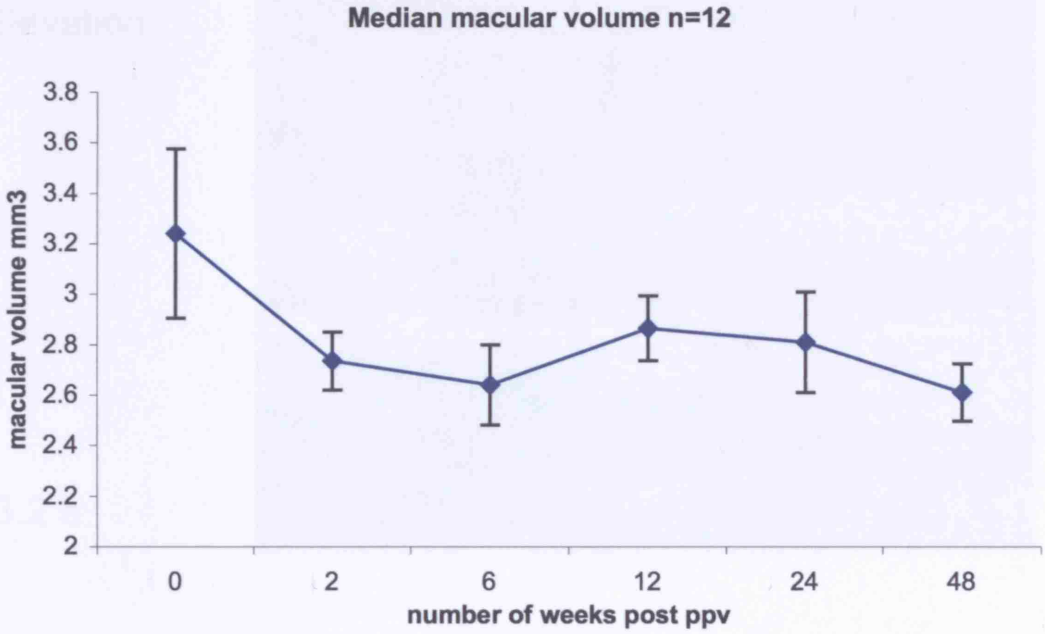
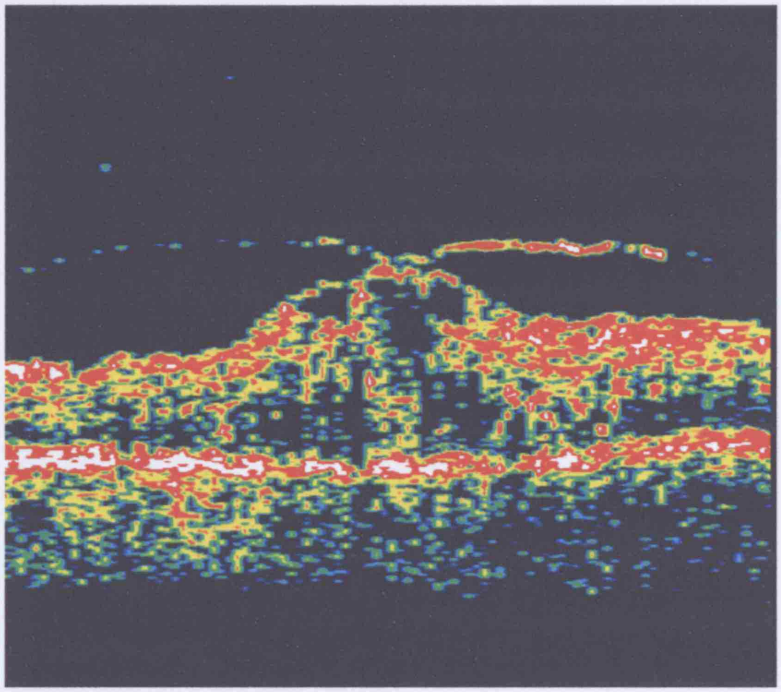


Fig 3.1

Figure 3.1 Macular structural changes post vitrectomy in the pilot series

A significant decrease (p=0.01) in the median foveal thickness at 12 months post pars plana vitrectomy from a baseline 334 mm (range 183 mm – 751 mm) to 280 mm (range 172-385 mm). A significant decrease (p=0.01) in the median macular volume at 12 months post pars plana vitrectomy from a baseline 3.24 mm^3 (range 2.13 – 6.42 mm^3) to 2.61 mm^3 (range 172-385 mm).

Group 1:
Dome-shaped
elevation



Group 2: Diffuse-
Low Elevation

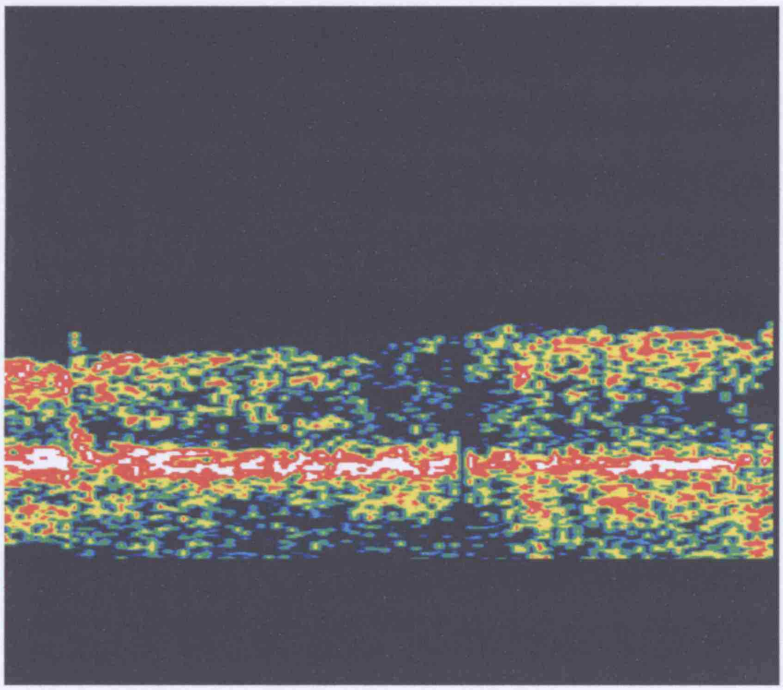
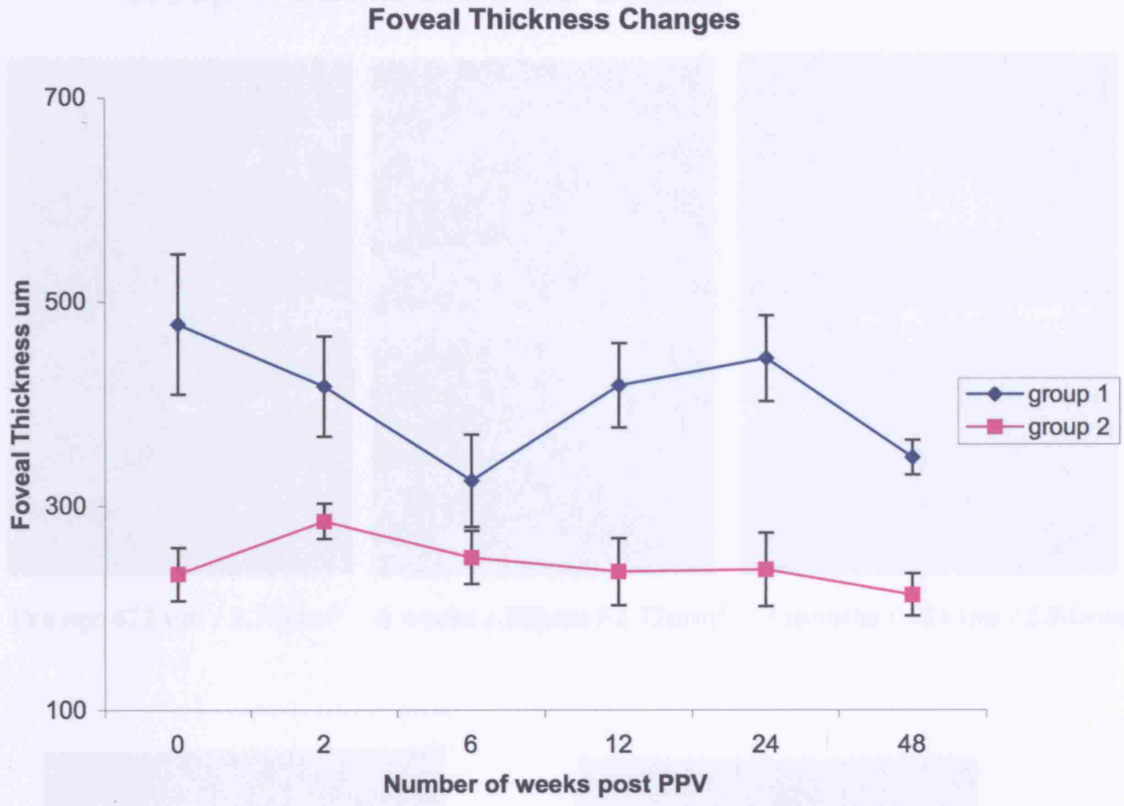


Fig 3.2 a

Figure 3.2a The two OCT defined macular profiles.

Group 1 (posterior hyaloid face still attached at the fovea) and Group 2 (no OCT evidence of posterior hyaloid separation). Baseline median foveal thickness was 233 mm (range 198-374mm) and macular volume was 2.7 mm^3 (range 2.13-3.52 mm^3). The difference in the pre-operative foveal thickness and macular volumes between the two groups was statistically significant (p=0.007).

In Group 1 the median foveal thickness showed an initial dramatic improvement over the first 6 weeks post surgery but then improvement was variable and gradual over the 12 months from a baseline median of 478 to a median of 348μm (**see Figure 3.2b**). For Group 2 there was little change in the foveal thickness throughout the postoperative period (from a median of 233 to a median of 213 μm) (**see Figure 3.2b**). The difference in foveal thickness between the two groups at 12 months postoperatively was significant (p=0.003). The macular volume changes for Group 1 and Group 2 reflected the foveal thickness changes post surgery (**figure 3.2b**). Unlike foveal thickness, the difference in the 12-month post-operative volumes between the two groups was not significant (p=0.06). **Figure 3.2c** demonstrates the change in the OCT image of a patient in Group 1 (dome-shaped) post surgery and **Figure 3.2d** shows the corresponding clinical appearance: the late image of the FFA post surgery demonstrates a decrease in the hyperfluorescence and leakage compared to preoperative appearance, indicating a reduction in macular oedema which is in agreement with the OCT images showing a reduction in the macular thickness. **Figures 3.2e and 3.2f** are the corresponding OCT and clinical appearances post vitrectomy in a patient with the diffuse-low elevation (Group 2) macular thickness: the late image of the FFA post surgery also demonstrates a reduced hyperfluorescence and leakage as does the OCT images showing improvement in the structural appearance of the macula.



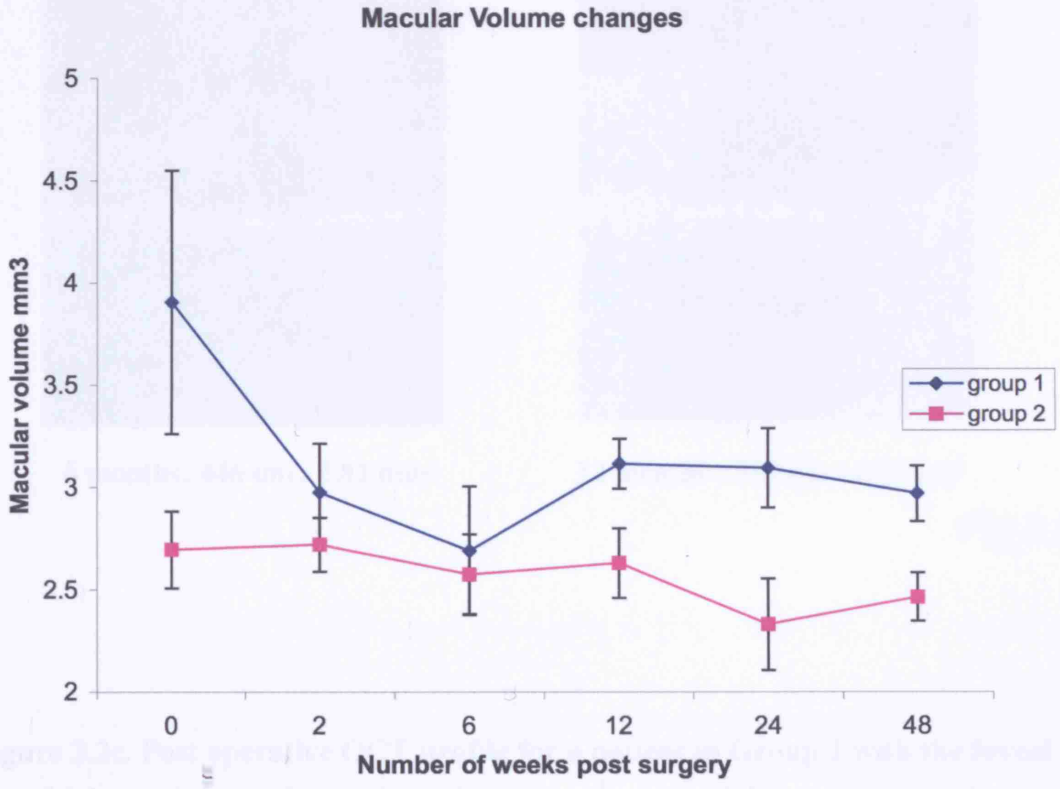
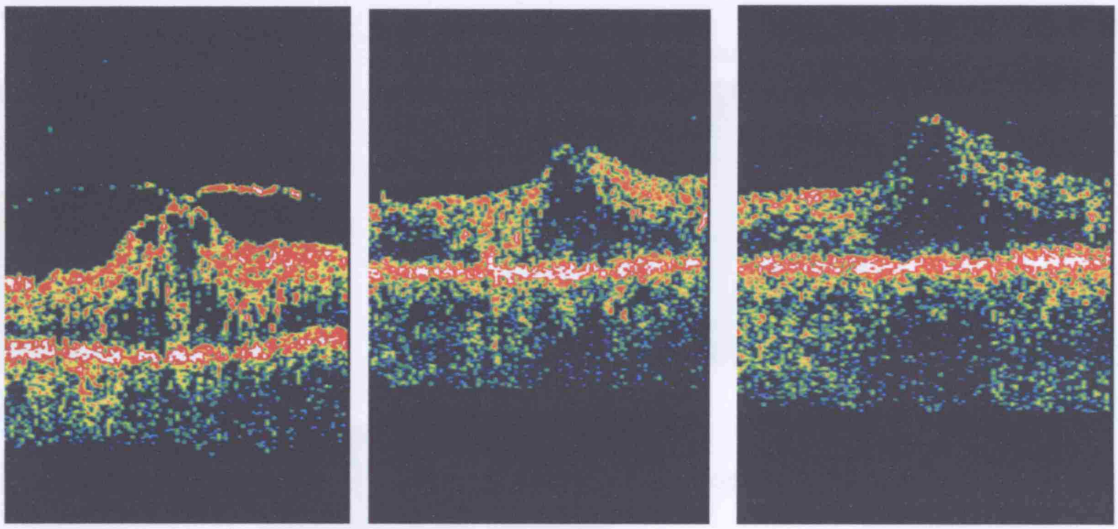


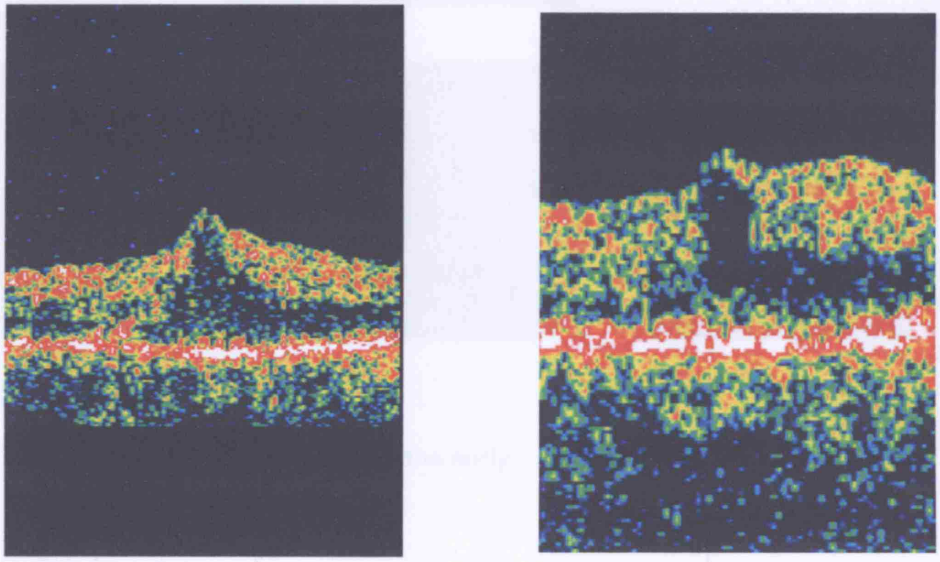
Fig 3.2b

Figure 3.2b. The different structural changes seen in these two groups.

Group 1: DOME SHAPED GROUP



Pre op: 472 um / 3.74mm^3 6 weeks : 322um / 2.72mm^3 3 months : 421 um / 2.94mm^3



6 months: 446 um / 2.82 mm^3 12 months : 310 um / 2.53mm^3

Fig 3.2c

Figure 3.2c. Post operative OCT profile for a patient in Group 1 with the foveal thickness (μm) and macular volume (mm^3): an initial large decrease is seen and then variability of response until a final decrease is seen at 12 months post surgery

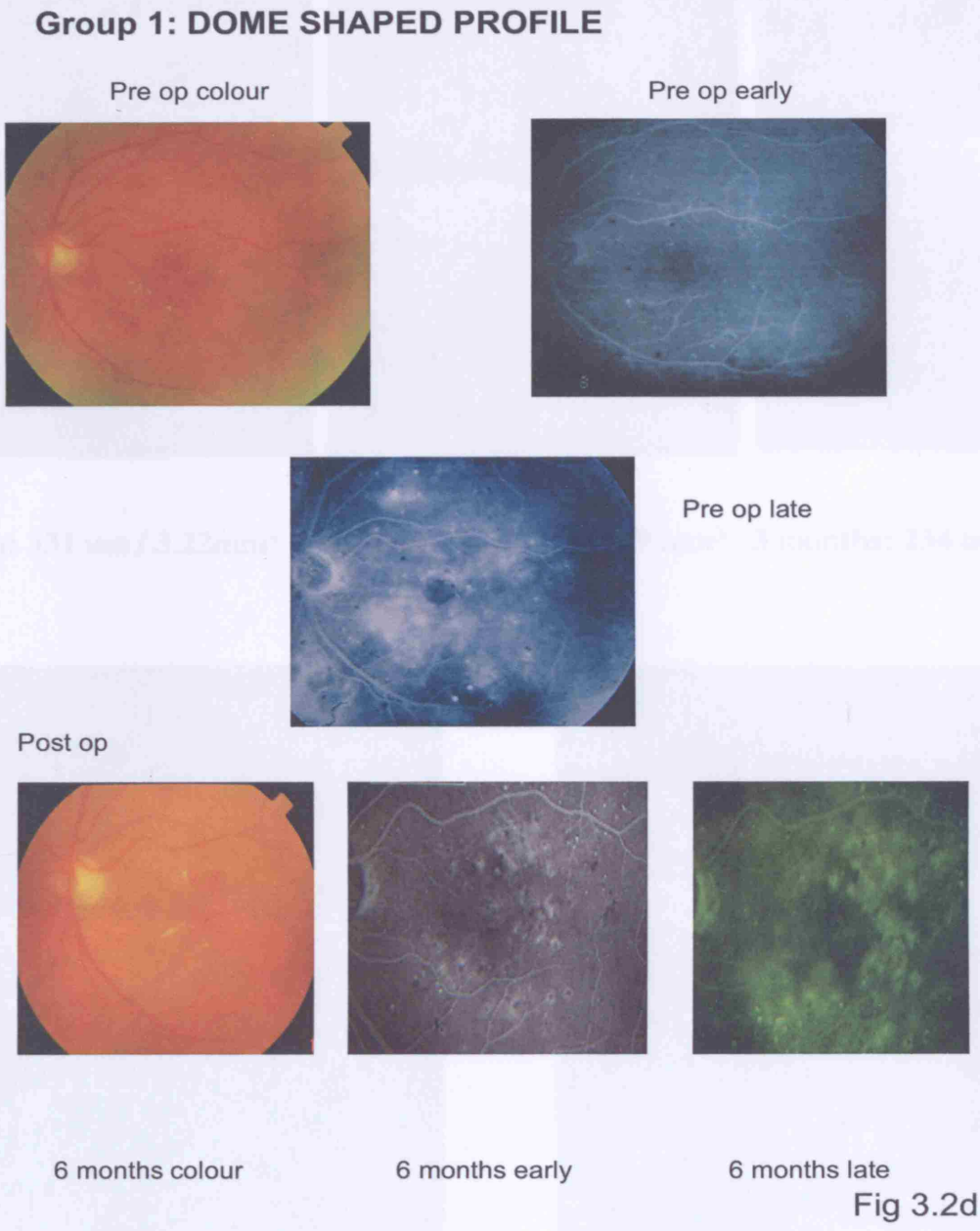
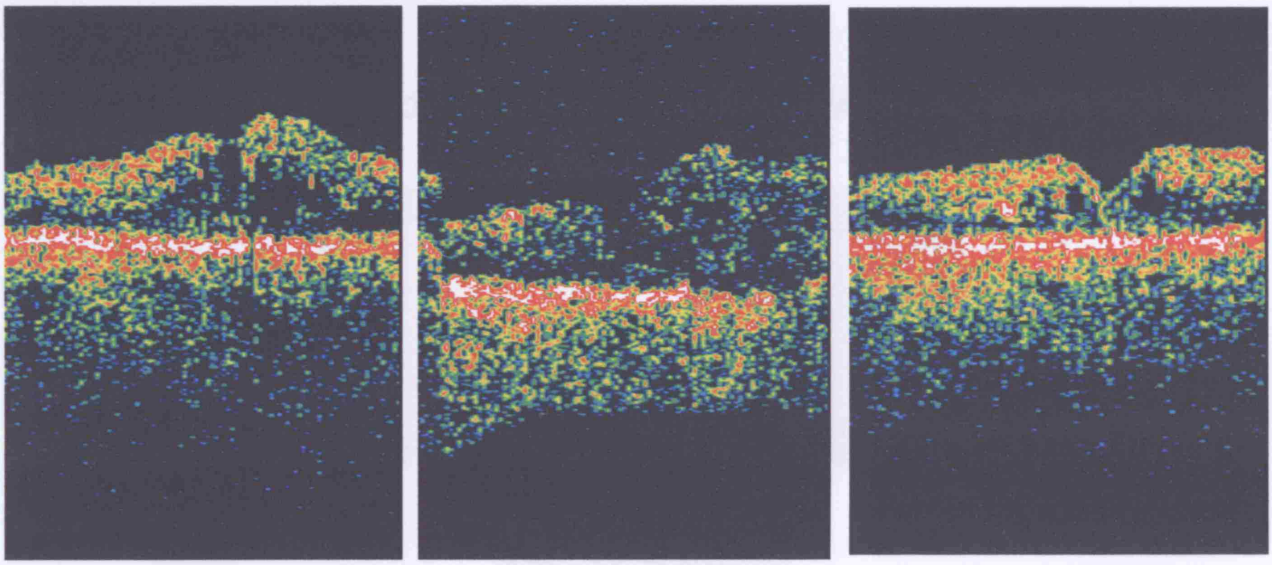


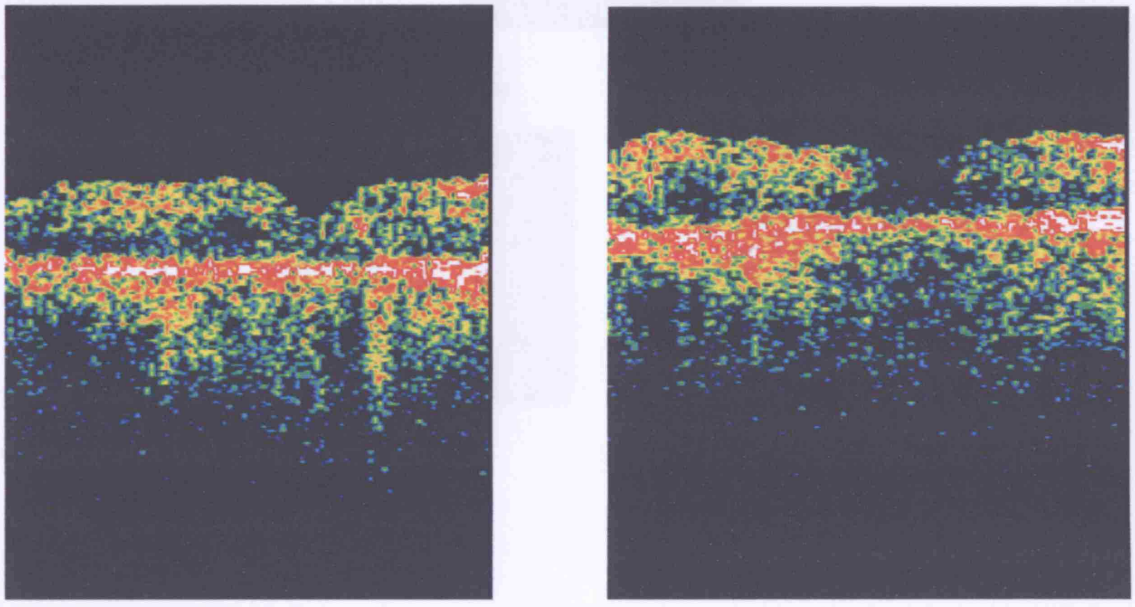
Figure 3.2d. Clinical and FFA appearance post surgery for a patient in Group 1:

An improvement in the clinical appearance of the macula on the colour fundus image and a reduction in late leakage post surgery compared to pre operative appearance. The 6 month and 12 month images were similar for this patient.

Group 2: DIFFUSE-LOW ELEVATION GROUP



Pre op: 331 um / 3.22mm^3 **6 weeks : 233 um / 2.39 mm^3** **3 months: 234 um / 2.64 mm^3**



6 months: 257 um / 2.33 mm^3 **12 months: 202 um / 2.35mm^3**

Fig 3.2e

Figure 3.2e. OCT profile of a patient in Group 2

Macular appearance showing a gradual improvement in the macular structural indices.

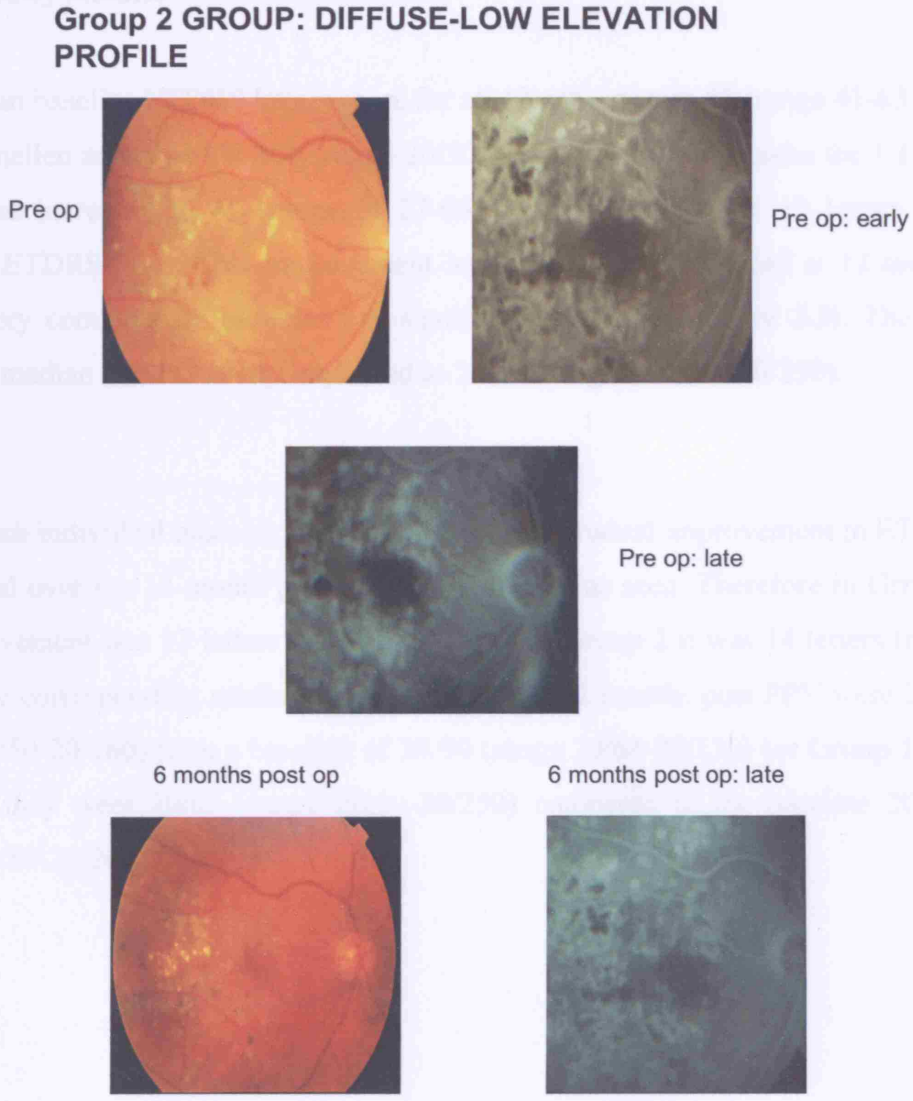


Fig 3.2f

Figure 3.2f. The clinical and FFA appearance of a patient in Group 2 post surgery. Again an improvement in the macular appearance of the colour image and a reduction in the late leakage on FFA post surgery is shown.

Visual Acuity Results

The median baseline ETDRS letters score for all 12 patients was 52 (range 41-63) or a median Snellen acuity of 20/ 100 (range 20/50 to 20/200). At 12 months the ETDRS letters read increased to 65 (range of 27-68), an improvement of 13 letters or 2 complete ETDRS lines. This improvement in the ETDRS letters read at 12 months post-surgery compared to baseline was significant (p=0.037) (**figure 3.3**). The best corrected median Snellen acuity improved to 20/56 (range 20/50 to 20/250).

Within each individual macular profile group a small gradual improvement in ETDRS letters read over the 12-month post operative period was seen. Therefore in Group 1 the improvement was 13 letters (range 7-15) and for Group 2 it was 14 letters (range 5-11). The corresponding median Snellen acuities at 12 months post PPV were 20/50 (range 20/50-20/160) from a baseline of 20/90 (range 20/64-20/126) for Group 1. For Group 2 they were 20/63 (range 20/50-20/250) compared to the baseline 20/112 (range 20/50-20/200).

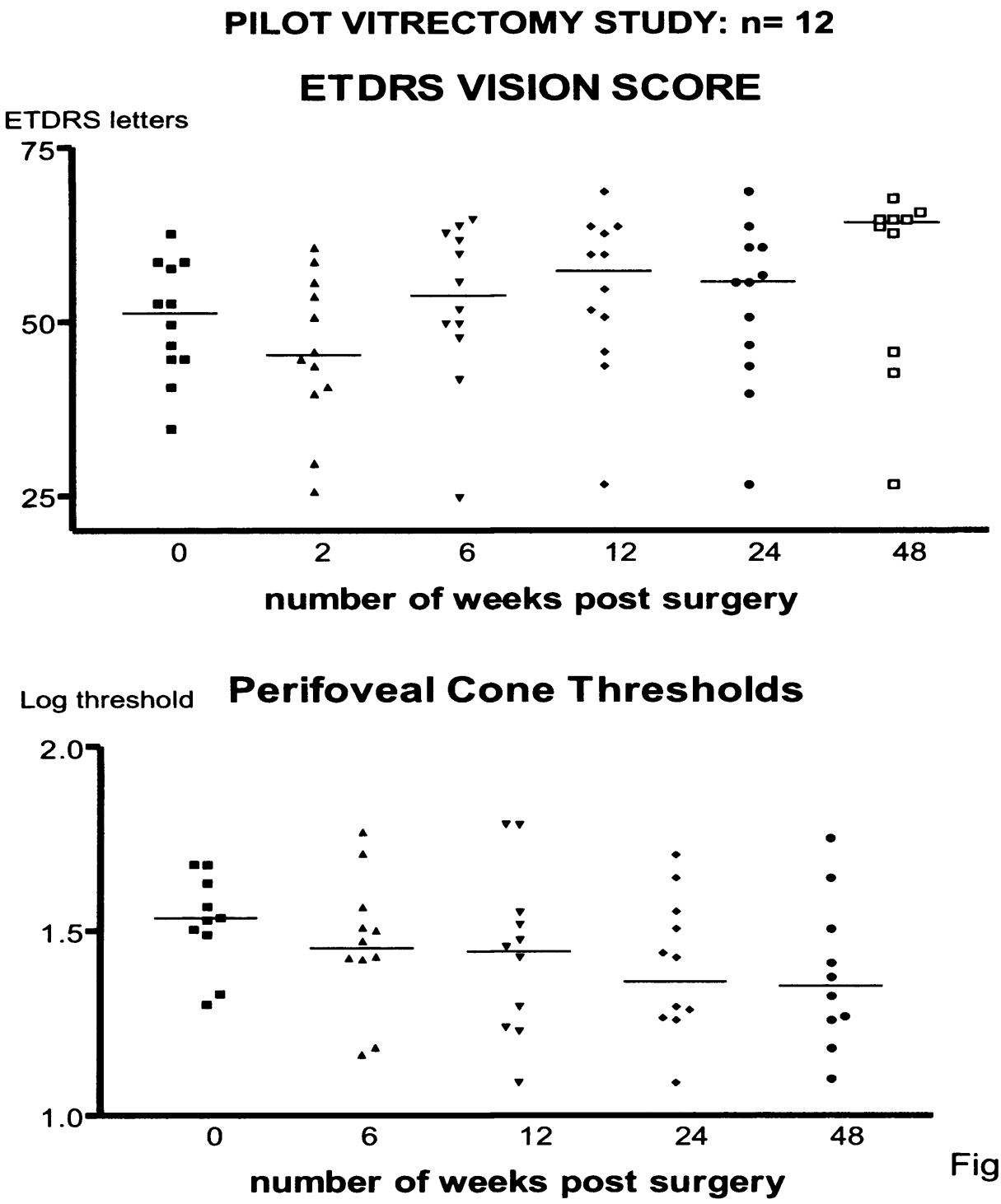


Fig 3.3

Figure 3.3. The functional improvement after vitrectomy

Improvement in the ETDRS and fine matrix mapping (perifoveal cone function): at 12 months ETDRS vision had significantly improved compared to baseline (p=0.037) as did fine matrix mapping (p=0.02).

Fine Matrix Mapping (Cone Threshold) Results.

Similar to the improved visual acuity, there was a statistically significant improvement in the perifoveal cone thresholds at 12 months compared to baseline for all 12 patients ($p=0.02$) (**Figure 3.3**). However even though each group showed similar improvement in the cone thresholds during the same time period, neither individual group showed statistical improvement ($p>0.05$).

Overlay of the cone thresholds and retinal thickness in the perifoveal 9 x 9 degree field, demonstrates 3 main types of post vitrectomy changes in the perifoveal region at the 12 months post surgery time points:

- Regional improvement in retinal thickness and cone threshold occurred in 7 eyes (**see Figure 3.4 a** where there is an improvement in the temporal region)

- Widespread improvement throughout the whole field tested for both thickness and threshold were seen in 1 eye (**see Figure 3.4 b**)

- No improvement in either parameter was seen in 4 eyes.

All 3 types of change were seen in both anatomical groups described above. Regional improvements were mainly seen in the nasal, temporal and superior quadrants.

Multiple linear regression analysis showed statistically significant association between the percentage improvement in the foveal thickness and macular volume at 12 months ($p=0.002$). Neither the final visual acuity nor the perifoveal cone thresholds showed significant correlations with the structural parameters ie foveal thickness or macular volume ($p>0.05$).

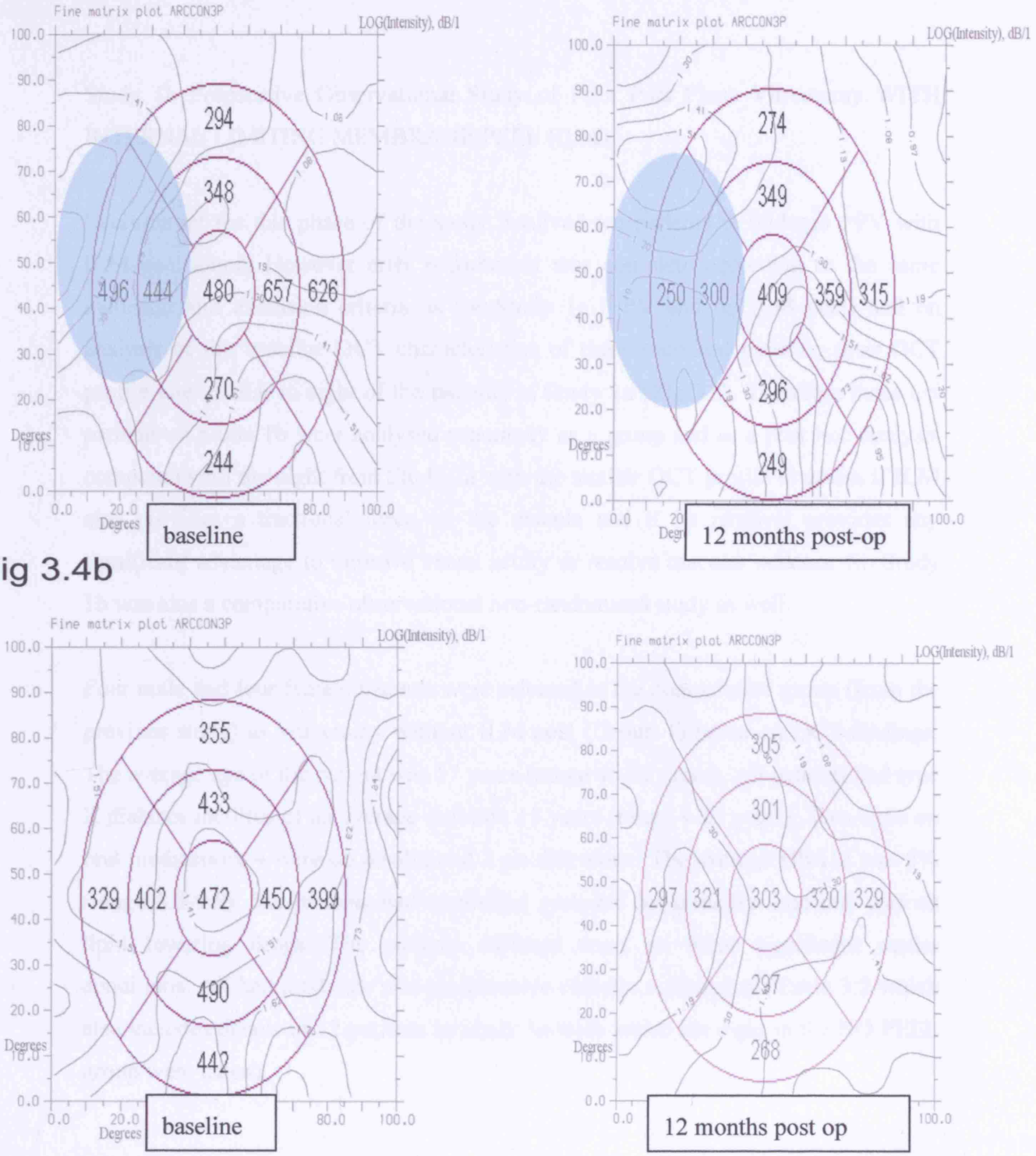


Figure 3.4 Overlay of the structural (foveal thickness in the paramacular region) on the functional (perifoveal cone thresholds) response post vitrectomy.

A) demonstrating corresponding regional improvement in both parameters

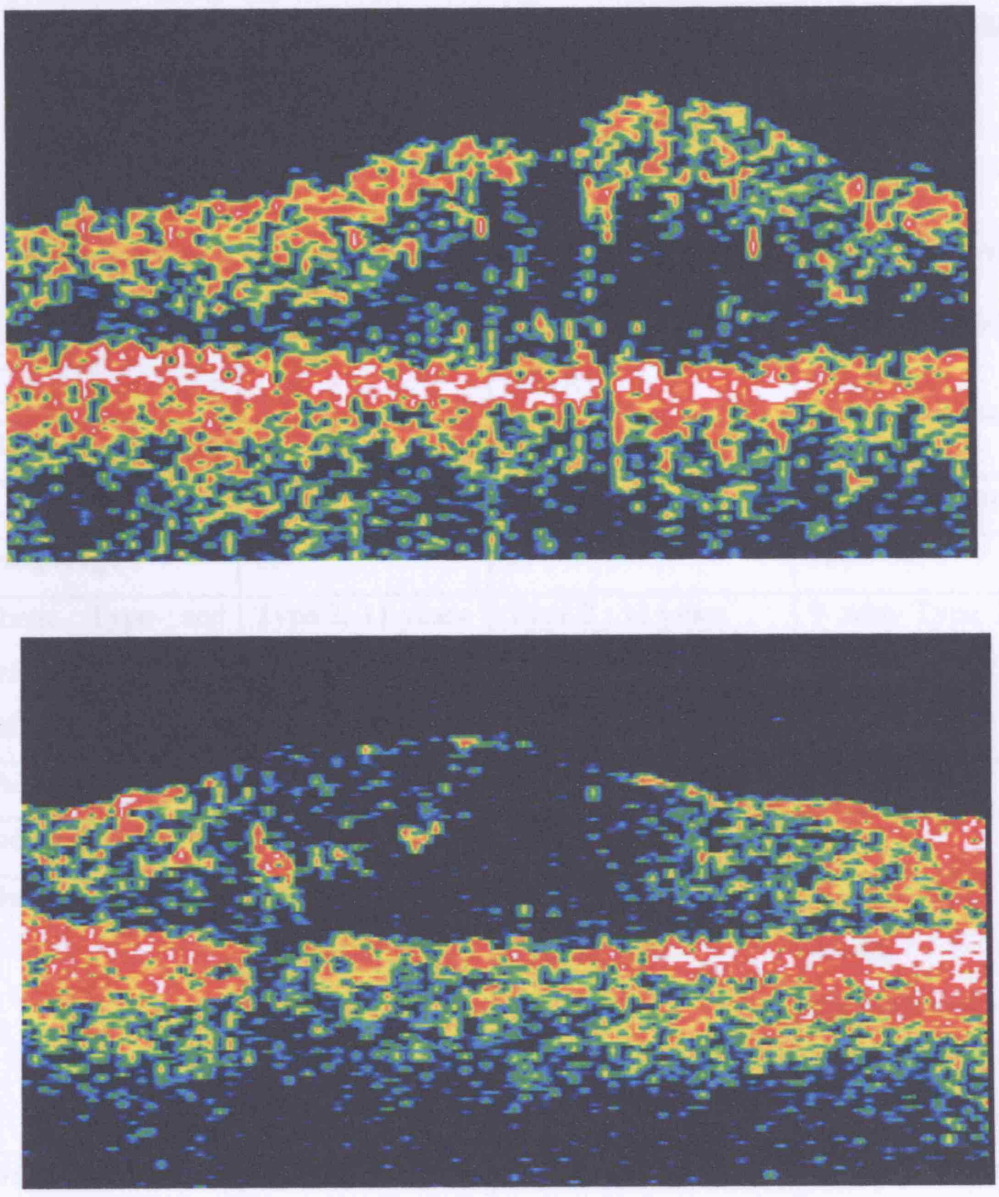
B) demonstrating corresponding global improvement in both parameters

Study 1b Propsective Observational Study of Pilot Pars Plana Vitrectomy WITH INTERNAL LIMITING MEMBRANE PEEL (ILM).

Recruitment for this phase of the study involved ten patients to undergo PPV with ILM peel alone. However after recruitment was complete according to the same inclusion and exclusion criteria as for Study 1a (PPV and no ILM peel) and on analysis of the baseline OCT characteristics of these recruited patients, their OCT profile was similar to eight of the patients in Study 1a (**Fig 3.5).** Therefore these ten patients of Study 1b were analysed separately as a group and as a post hoc analysis compared with the eight from Study 1a with the similar OCT profile to assess if ILM also provides a tractional force on the macula and if its removal provides any significant advantage to improve visual acuity or resolve macular oedema. So Study 1b was also a comparative observational non-randomised study as well.

Four male and four female patients were selected as the comparative group (from the previous study) as vitrectomy without ILM peel (Group 1) based on OCT findings. The average age of the patient was 57 years (range 46-72 years). All patients had type II diabetes mellitus of an average duration 11 years (range 4-23 years). Two were on oral medication; 4 were on insulin and 2 on diet alone. The average HbA1c was 8% (range 5.8-9.9). Six patients had controlled systemic hypertension and one was on lipid lowering drugs. The patients suffered from no other significant ocular conditions. All had moderate non-proliferative diabetic retinopathy (Table 3.2 which also includes details of 12 patients in Study 1a from which the eight in the NO PEEL group were taken).

Group 1 : no ILM peel



Group 2 :ILM peel

Fig 3.5

Figure 3.5. OCT of macular profile in the patients recruited into the two groups: ILM Peel and No Peel Groups.

A similar macular profile for both groups: diffuse low elevation

Table 3.2 Demographic and clinical data of the patients recruited into the ILM peel study and the comparative group (No Peel) with details of Study 1a from which the No Peel Group was selected.

	Study 1a	Study 1b NO PEEL COMPARATIVE GROUP	Study 1b ILM PEEL GROUP
Number	12	8	10
Sex	7 male; 5 female	4 male and 4 females	8 males; 2 females
Average Age	63	57	73
Diabetic Type and average years of duration	Type 2; 11 years	Type 2 ; 11 years	9 with Type 2; 1 with Type 1; 10 years
HbA1C (%)	8	8	10
Hypertension	9	6	4
Diabetic Retinopathy	All had moderate non-proliferative diabetic retinopathy	8 moderate non-proliferative	7 moderate to severe non-proliferative; 3 quiescent proliferative
Previous Macular Laser and average duration of macular oedema prior to surgery	3 laser treatments 15 months of oedema	3 laser treatments 15 months of oedema	3 laser treatments 15 months of oedema

Eight males and 2 females with an average age 73 (range 31-72) were recruited into the group of patients undergoing vitrectomy with ILM peel (Group 2). Nine patients had type II and one had type 1 diabetes mellitus, with an average duration of 10 years (range 2-27 years). Three patients were on oral hypoglycaemics and the remainder on insulin, and the average HbA1C was 10% (9.5-11%). Four patients were controlled

systemic hypertensives. Retinopathy was graded as moderate to severe non-proliferative in 7 patients and quiescent proliferative in three (Table 3.1).

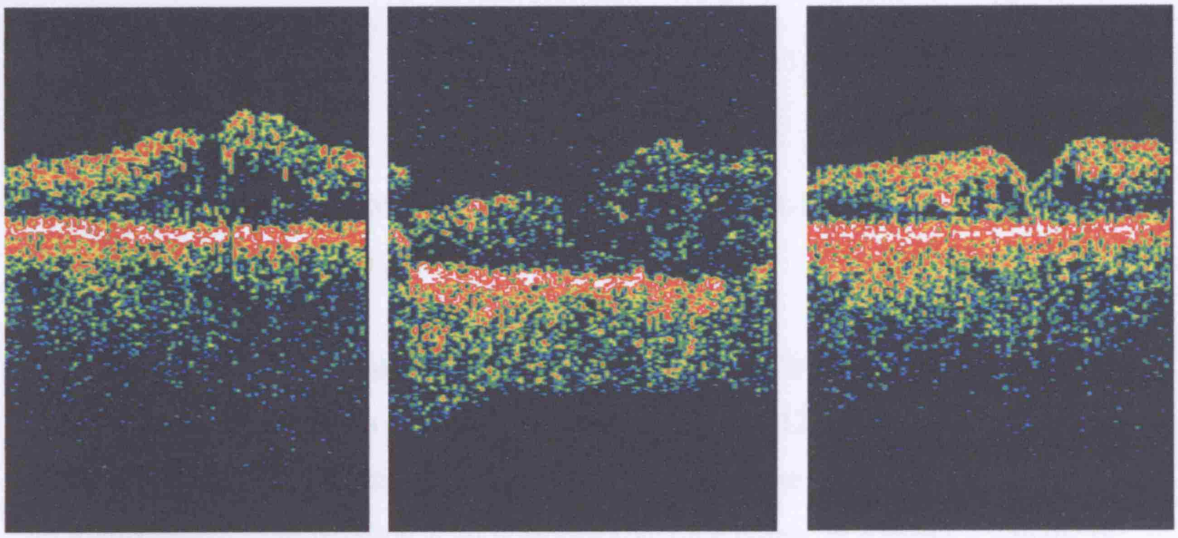
All patients recruited to this comparative study had macular oedema, which had been previously treated with macular argon laser treatment on average 3 times (range 1-5) and had been present for an average of 15 months (range 12-18 months) prior to surgery. There were no signs of macular ischaemia as assessed on FFA.

Per-operative assessment confirmed attached posterior hyaloid face in all cases. Posterior hyaloid separation was induced as part of the operative protocol in both groups of patients and the additional ILM peel in the second group. There were no complications resulting from the surgery. The patients were operated on consecutively by the same surgeon (ZG)

OCT findings

Comparative Group (No ILM peel) (n=8)

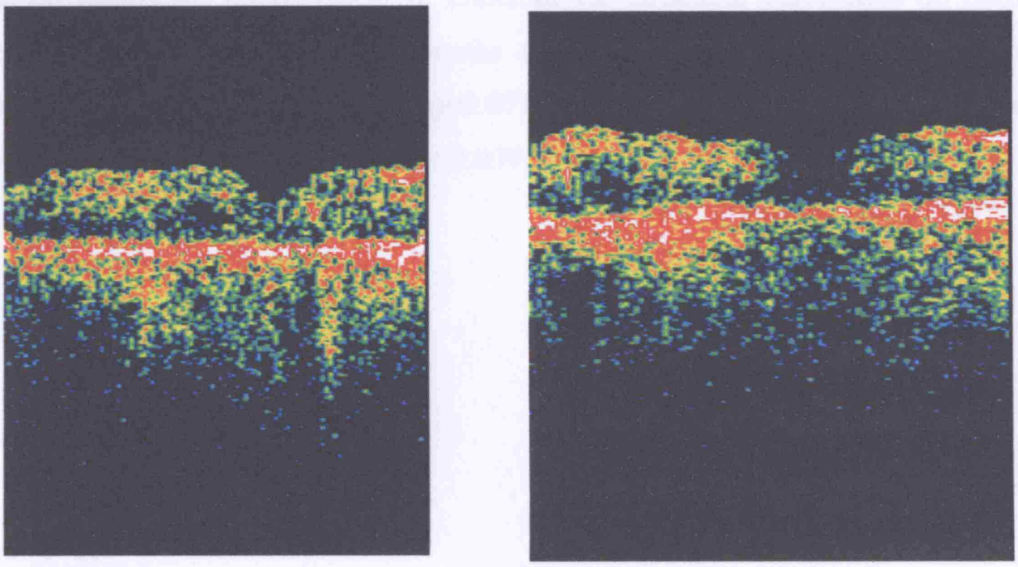
The median foveal thickening for all 8 patients was 233 μm (range 180 μm – 374 μm) (normal range 126-180 μm) and the median macular volume was 2.7 mm^3 (range 2.13 – 3.52 mm^3, normal < 1.66 mm^3). Twelve months after surgery, the foveal thickness in the 8 patients had decreased to a median of 213μm (range 172-250μm) whilst the median macular volume decreased to 2.47 mm^3 (range 2.06-2.86 mm^3) ($p>0.05$) (**Fig 3.6, OCT profile of a single patient representative of this group**).



Pre op: 331 um / 3.22mm^3

6 weeks : 233 um / 2.39 mm^3

3 months: 234 um / 2.64 mm^3



6 months: 257 um / 2.33 mm^3

12 months: 202 um / 2.35mm^3

Fig 3.6

Figure 3.6 OCT profile illustrating macular structural improvement in one patient who underwent vitrectomy without ILM peel

ILM peel group (n=10)

Baseline median foveal thickness was 400 μm (range 256-509 μm) with a macular volume of 3.28 mm^3 (range 2.33-4.8 mm^3).

Post operative OCT showed an improvement in the foveal thickness at 6 weeks (259 μm, range 142-643), then a gradual worsening at 3, 6 and 12 months (275 μm, range 173-844 μm) seen at these time points (**see Figure 3.7**). The macular volume showed an improvement from baseline at all postoperative time points: at 6 weeks a volume of 2.59 mm^3 (range 1.8-4.8 mm^3), at 12 and 24 weeks 2.83 mm^3 (range 1.89-5.62) and at 48 weeks (12 months) after surgery a volume of 2.69 mm^3 (range 1.67-4.23 mm^3) (**see Figure 3.8, OCT profile of a single patient representative of this group**). There was no significant improvement in either of the structural parameters up until 6 months post-surgery but by 12 months after vitrectomy the decrease in foveal thickness approached significance (p=0.07) whilst the decrease in macular volume showed a significant improvement (p=0.039).

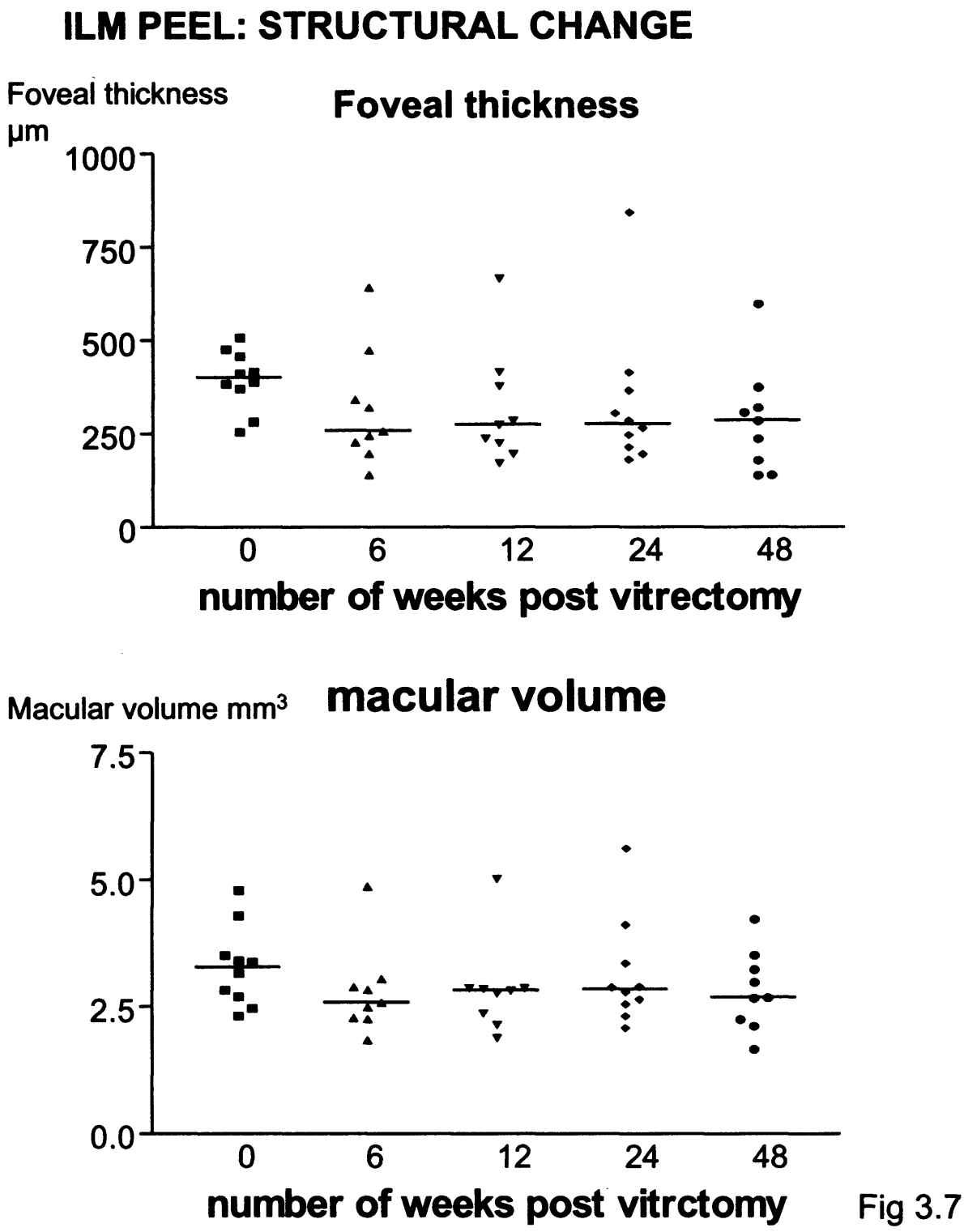


Fig 3.7

Figure 3.7 Structural improvements after vitrectomy with ILM peel.
Only the macular volume showed a significant improvement at 12 months compared to baseline (p=0.039).

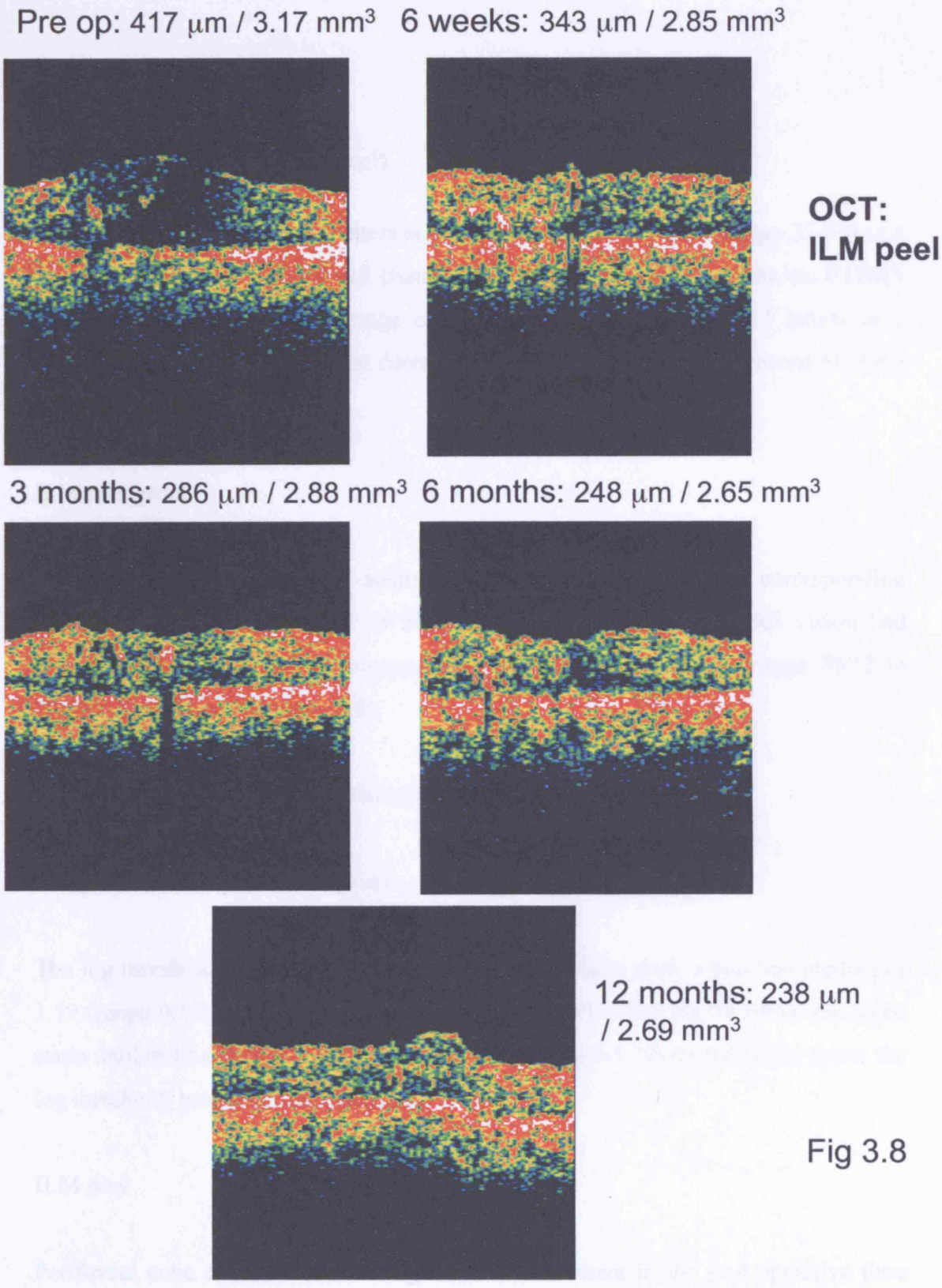


Fig 3.8

Figure 3.8 OCT profile illustrating macular structural improvement in one patient who underwent vitrectomy with ILM peel

Visual Acuity Results

Comparative Group (No ILM peel)

The median baseline ETDRS letters score for all 8 patients was 52 (range 35-63) or a median Snellen acuity of 20/ 112 (range 20/50 to 20/200). At 12 months the ETDRS letters read increased to 65 (range of 27-68), an improvement of 13 letters or 2 complete ETDRS lines. The best corrected median Snellen acuity improved to 20/63 (range 20/50 to 20/250).

ILM peel group

Pre operative median ETDRS acuity was 65 (range 50-75) with a corresponding Snellen of 20/50 (range 20/32 to 20/125). By 12 months the ETDRS vision had improved by 2 letters or a corresponding Snellen vision of 20/45 (range 20/32 to 20/160) ($p>0.05$) (**see Figure 3.9**).

Fine Matrix Mapping (Cone Threshold) Results.

Comparative Group (No ILM peel)

The log threshold of cone function does initially decrease from a baseline median of 1.59 (range 0.8-1.68) to 1.49 (0.7-1.57) at 6 weeks. Then the log threshold remained static until at 12 months it further decreased to 1.44 (0.6-1.76) ($p=0.07$) (the lower the log threshold, better the cone function)

ILM peel

Perifoveal cone function showed a gradual improvement in the post-operative time period where at 6 months a median level of 1.14 log thresholds (range 0.8-1.5) with a baseline of 1.4 log thresholds (range 0.9-2.9). At 12 months the log threshold was 1.2 (range 0.8-1.7) but these improvements were again not significant ($p>0.05$) (**see Figure 3.9**).

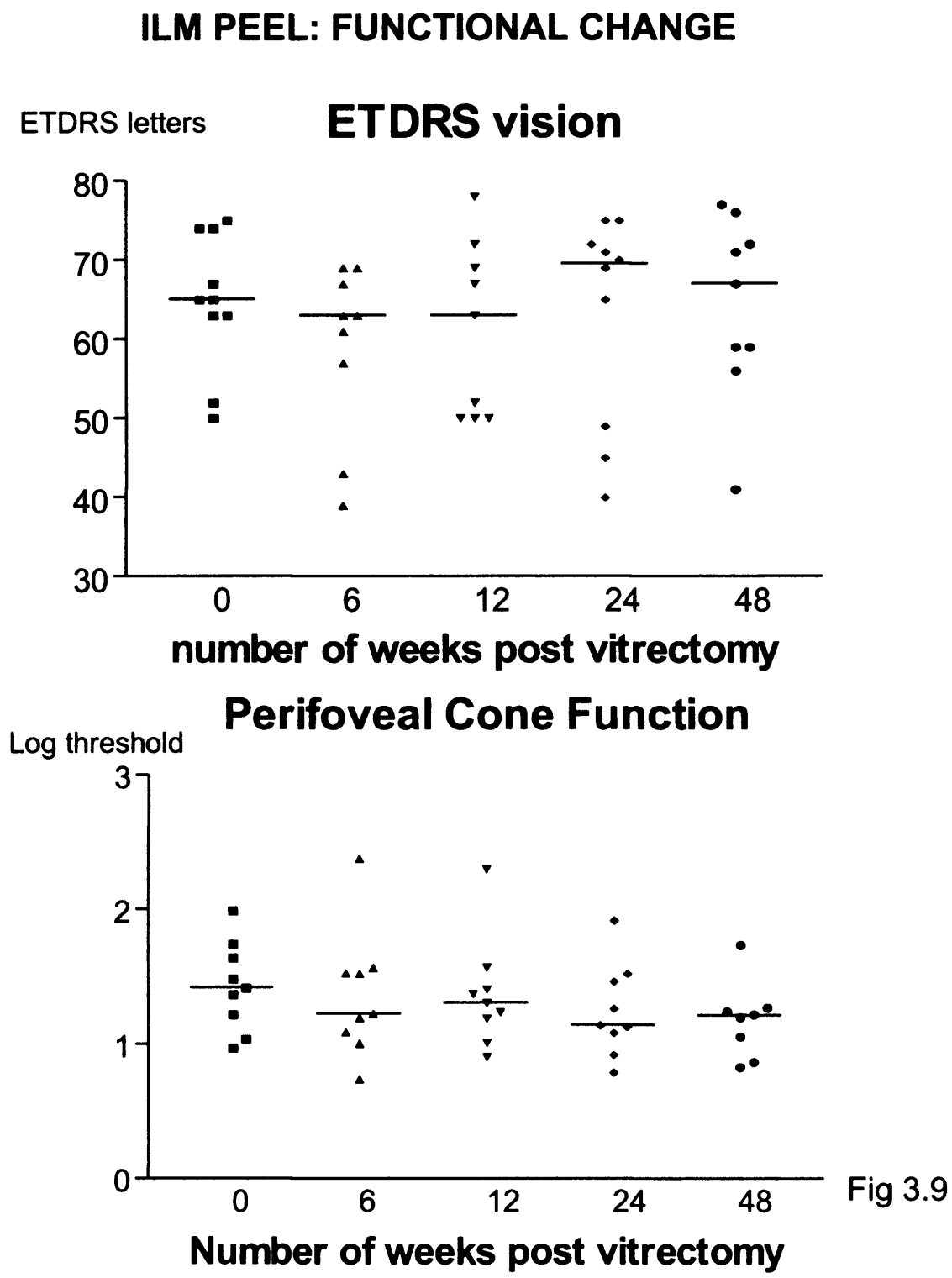


Figure 3.9 Functional changes after vitrectomy with ILM peel.

A non-significant improvement in visual acuity and perifoveal cone function at 12 months compared to baseline.

Comparative post hoc analysis between the no peel group and the peel group (see Table 3.3) (* denotes the only 12 month significant improvement compared to baseline p=0.039).

Comparing these two groups, the peel group had a significantly better ETDRS vision at baseline compared to the no peel group (p=0.024) which may explain the limited improvement in vision at 12 months in this group compared to the 13 letter improvement in the No Peel group 12 months after surgery. However despite the cone function showing some improvement for both Peel and No Peel groups, this improvement was small and non-significant when compared to baseline values. Furthermore the foveal thickness and macular volume showed improvements for both groups but only in the macular volume at 12 months in the Peel group was the improvement significant compared to its baseline volume. Both groups showed improvement in fundal appearance (**see Figure 3.10 fundal image of a single patient representative of each group**). However on inter-group analysis, the patients in the no peel group showed a trend towards a greater improvement for both structural indices 12 months after surgery (**see Figure 3.11**), whilst neither group showed a particular advantage in the improvement of the ETDRS letters read post surgery (**see Figure 3.12**).

Table 3.3 Functional and structural results comparing No ILM Peel with ILM Peel groups.

	NO ILM PEEL (n=8)		ILM PEEL (n=10)	
	BASELINE	12 MONTHS	BASELINE	12 MONTHS
Median foveal thickness (μm)	233	213	400	275
Median macular volume mm^3	2.7	2.47	3.28	2.69*
Median ETDRS vision (letters read)	52	65	65	67
Median perifoveal cone theshold (log units; the lower the value, the better the cone function)	1.59	1.44	1.4	1.2

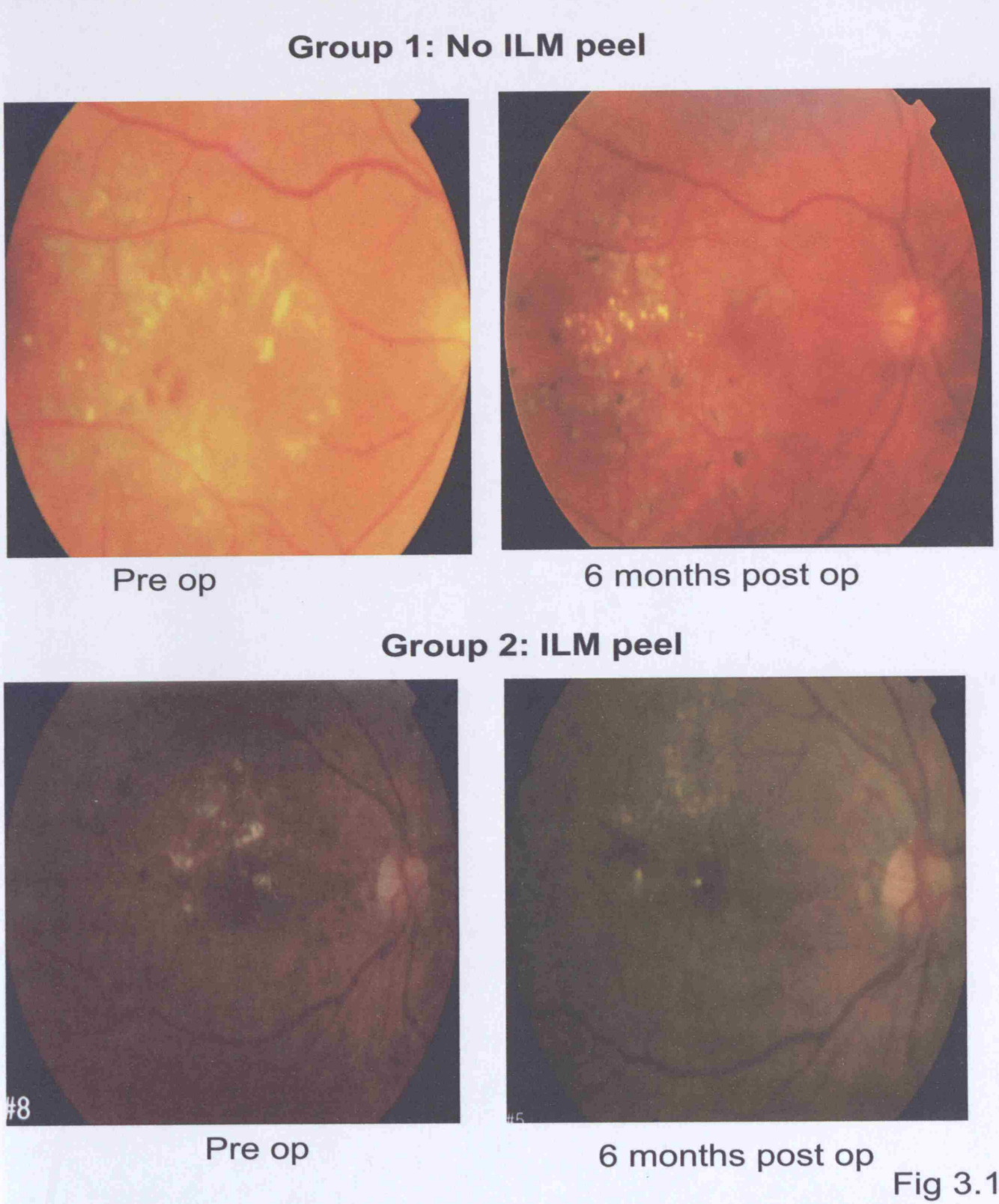
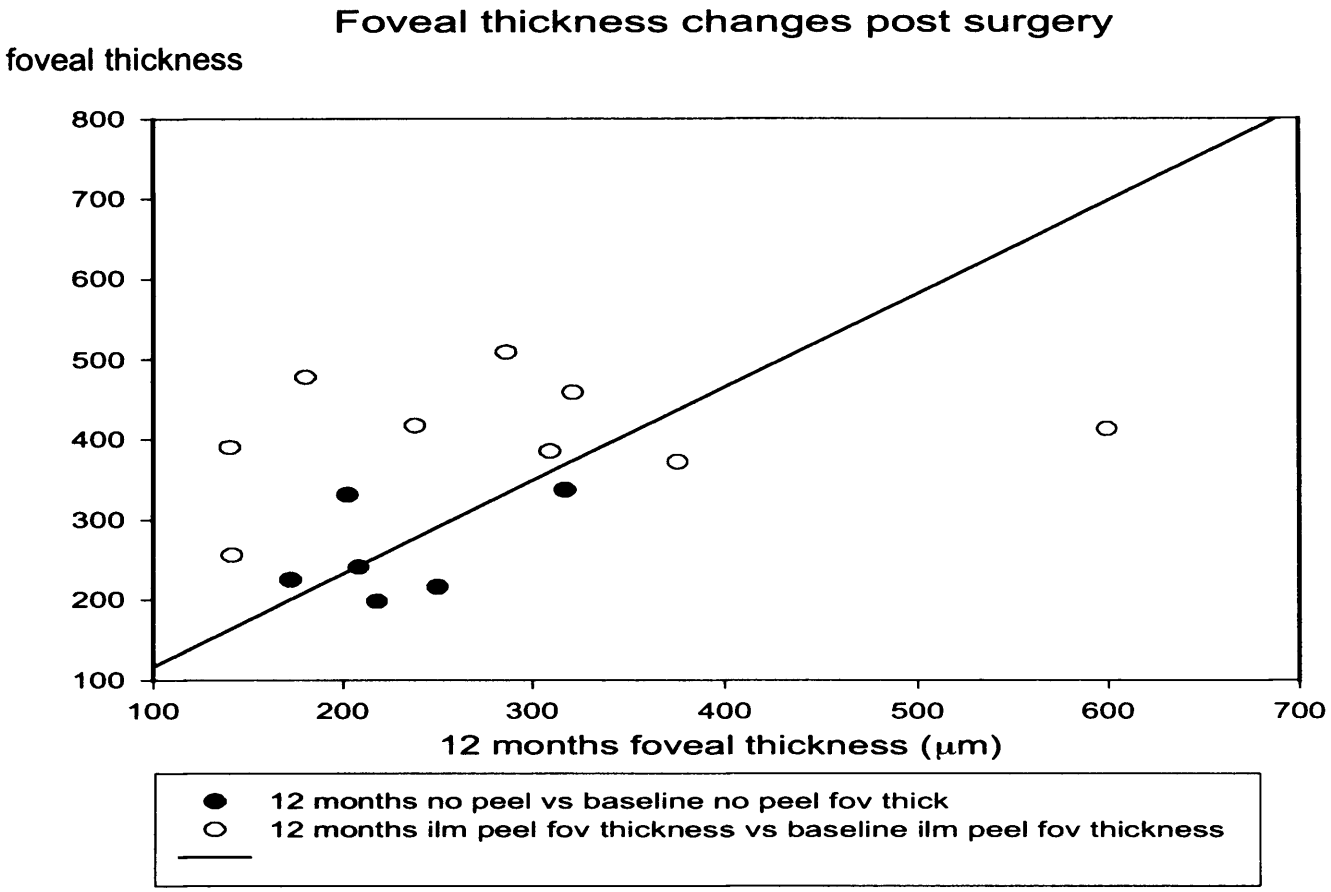


Figure 3.10 Fundal appearance post surgery comparing ILM peel and non-peel after vitrectomy.

Fundal appearance demonstrating decreased macular oedema at 6 months post surgery comparing NO PEEL and ILM PEEL. The 12 months appearance was similar.



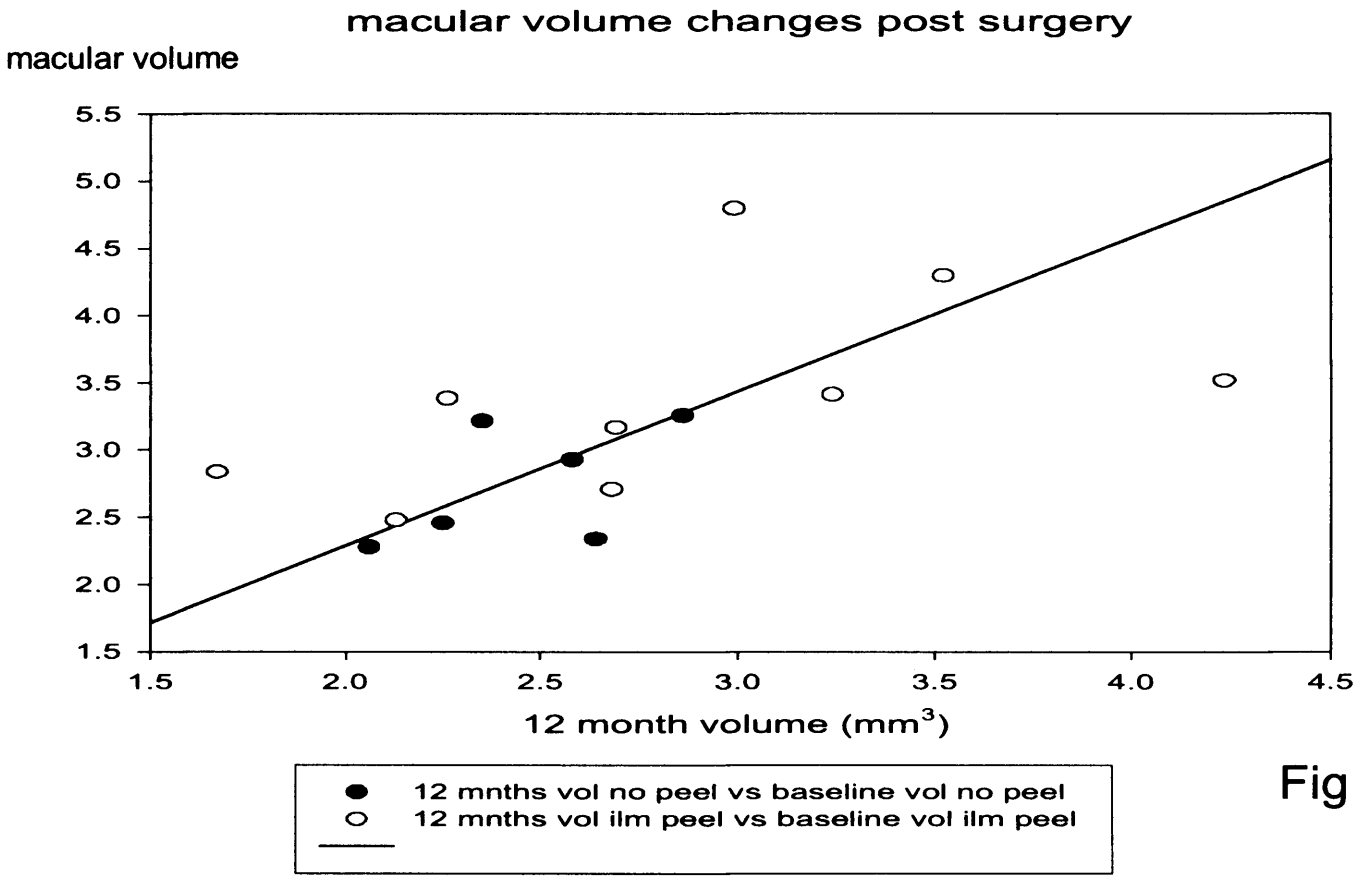


Fig 3.11

Figure 3.11 Scatter plot comparing the structural change between the ILM Peel and No Peel Groups.

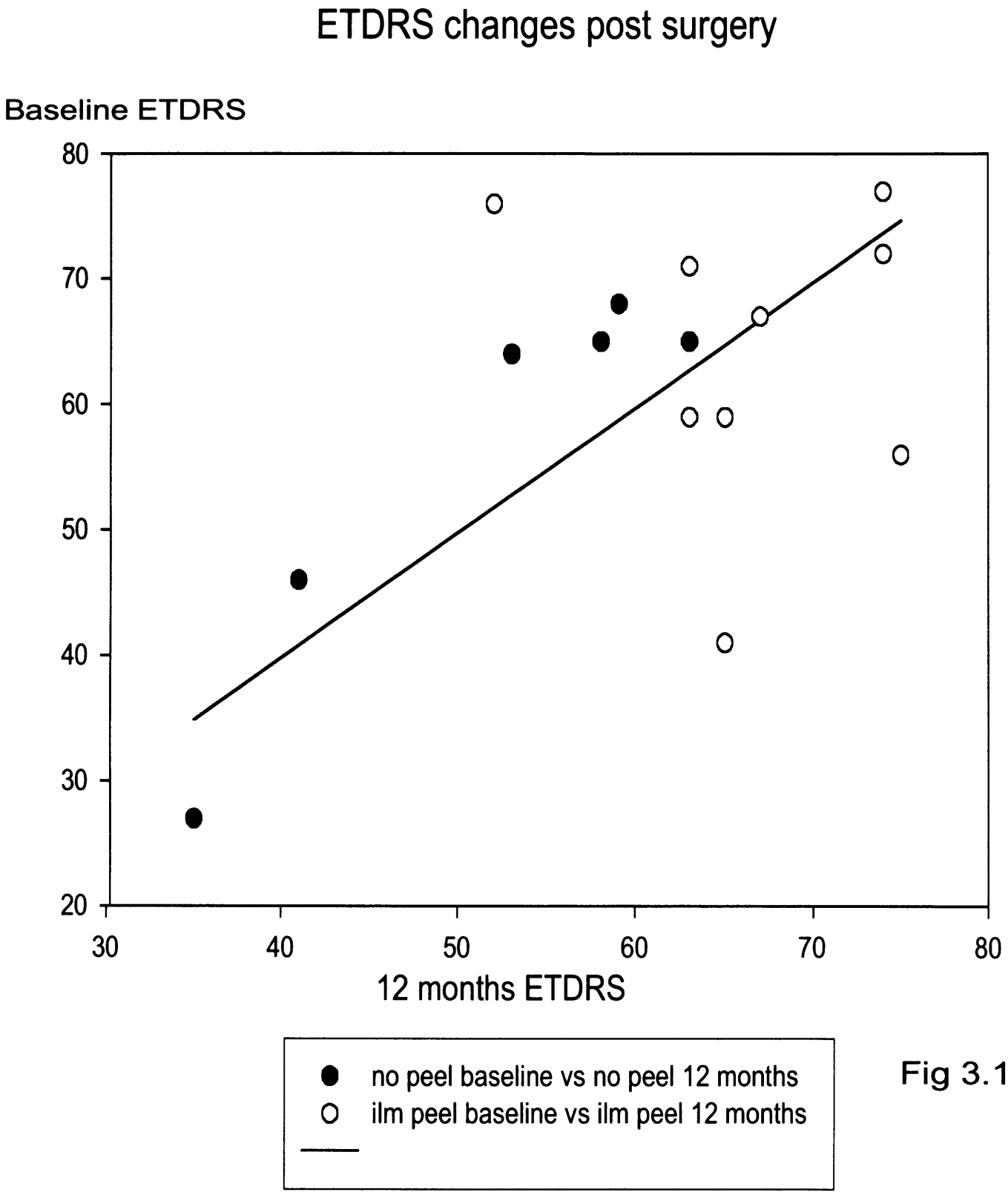


Fig 3.12

Figure 3.12 Scatter plot comparing the ETDRS vision between the ILM Peel and No Peel Groups. There was no significant difference in vision between the two groups at 12-post surgery

CHAPTER 3.1.2 Results of Pilot Randomised Control Trial: Pars Plana Vitrectomy vs. Macular Laser Treatment.

This study was conducted as a pilot study to determine the power and trial details for a larger more definitive study comparing the role of pars plana vitrectomy against standard macular laser treatment.

Twenty patients were equally randomised into each treatment arm. Seven patients in PPV arm completed the trial protocol. 3 patients failed to have treatment (one was involved in a serious road traffic accident prior to operation date, one had corrected visual acuity better than inclusion criteria, and one died prior to treatment). Eight patients in the laser arm completed the trial protocol. One patient was lost to follow-up, and one failed to have treatment (**see Figure 3.13**). No complications occurred in either treatment arm in any patient.

The average age of the patients who completed the protocol in the PPV group was 65 years (61-74 years) and four were male and three female. All had type 2 diabetes (five on insulin treatments), with an average of 15 years of diabetes (6-24 years) and an average HbA1c of 9.1% (8-10%) and all were controlled hypertensives. Clinically all had moderate-severe non-proliferative diabetic retinopathy with fovea-involving CSMO having been previously treated with macular laser on average 3 times (2-5) (Table 3.2)

Table 3.4. Table detailing the demographic and diabetic clinical details of patients recruited into the randomised trial.

	LASER ARM	VITRECTOMY ARM
Number	8	7
Average age (years)	64	65
Sex	2 male; 6 female	4 male; 5 female
Diabetic Type	Type 2	Type 2
Average years diabetes	14	15
HbA1C (%)	9	9.1
Hypertension	5	8
Diabetic Retinopathy	6 moderate-severe non-proliferative; 1 quiescent proliferative	All moderate-severe non-proliferative
Average number macular laser treatments	3	3
Average duration of oedema prior to recruitment (months)	14	14

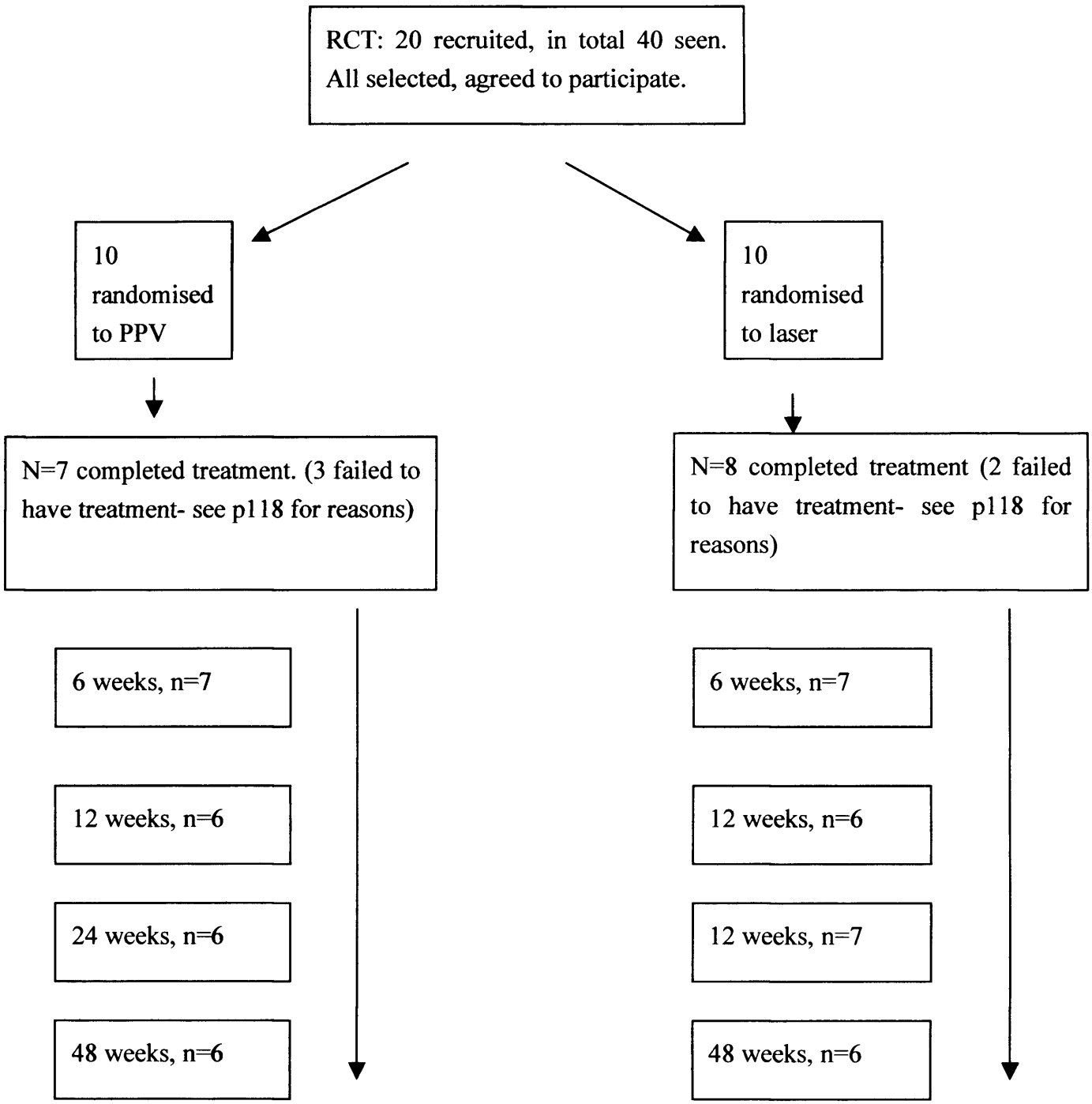


Figure 3.13 Flow chart depicting the course of the patients in the randomised control trial

The average age of the patients in the laser group who had completed the protocol was 64 years (50-71 years) and six were females and two male. All had type 2 diabetes (four on insulin treatment), with an average of 14 years of diabetes (5-22 years) and an average HbA1c of 9 % (9.5-11 %) and five had controlled hypertension. One had quiescent proliferative diabetic retinopathy whilst the others had moderate-severe non-proliferative diabetic retinopathy and all had fovea-involving CSME

having been previously treated with macular laser on average 3 times (1-5) (Table 3.2). All recruited patients had fovea-involving oedema for an average of 14 months (12-18 months) prior to recruitment. There was no significant difference in the distribution of factors such as sex and on insulin treatment (p=0.3147 Fisher's Exact test) between treatment groups.

Patients who were enrolled in this pilot trial adhered to the examination schedule. Of a total of 32 examinations expected for patients in the laser treatment arm from 6 weeks to 12 months after treatment, 29 (90%) were completed. Similarly for the PPV group of a total of 28 examinations expected over the same time period, 25 (89%) were completed.

Visual Acuity Results

The median baseline ETDRS letters read in the laser group was 62.5 (range 42-75) (Snellen equivalence 20/56, range 20/50 to 20/160). At 6 weeks it was 65 (range 50 to 79) (Snellen 20/50 to 20/100). At 3 months it had increased to 67 (range 53-79) (Snellen 20/50, range 20/40 to 20/100), at 6 months 66 (range 39-76) (Snellen 20/45, range 20/32 to 20/200) and at 12 months post-treatment it was 67.5 (range 61-80) (Snellen 20/45, range 20/32 to 20/63). No patients in the laser group suffered from moderate visual loss (MVL) at the 12 month final visit. (i.e. loss of 15 letters on the ETDRS chart) (**see Figure 3.14** and Table 3.3)

Table 3.5. Table detailing the final results comparing the two groups in the randomised study.

Median values	LASER		VITRECTOMY	
	Baseline	**12 months**	**Baseline**	**12 months**
ETDRS LETTERS	62.5	67.5	58	60
SNELLEN VA	20/56	20/45	20/80	20/56
FOVEAL THICKNESS	390	283	364	337
MACULAR VOLUME	3.55	2.77	3.23	2.81

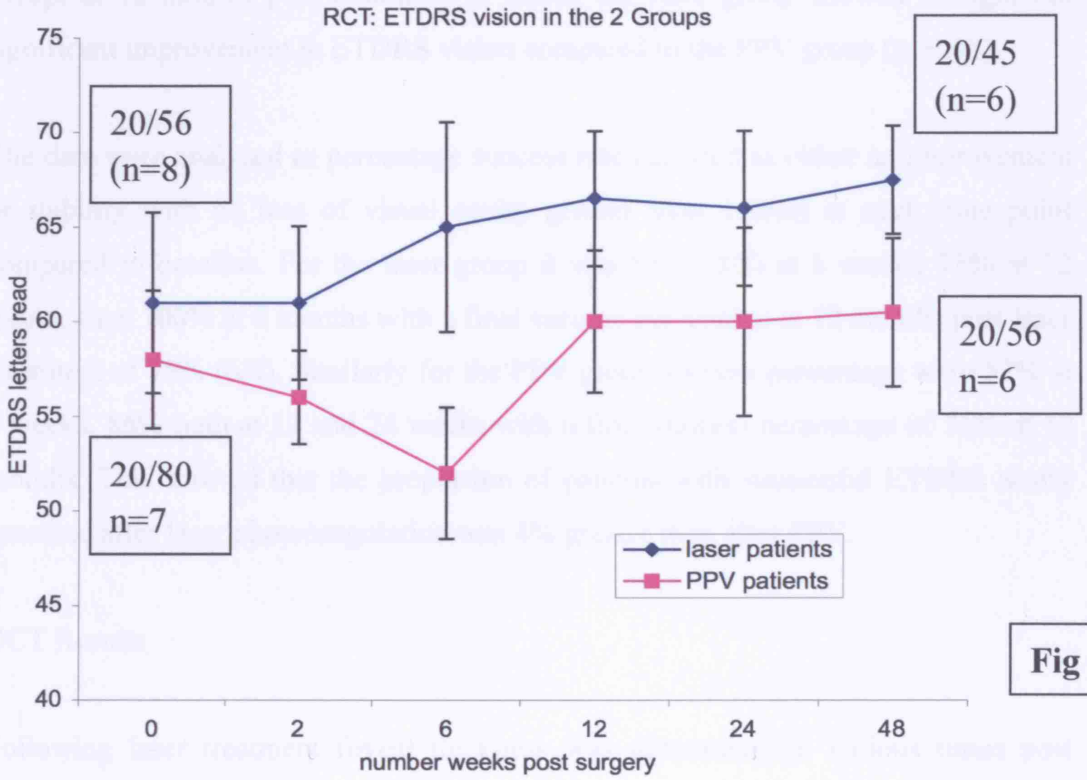


Fig 3.14

Figure 3.14 Comparing ETDRS vision between the two groups in the RCT after treatment.

Line graph demonstrating the change in vision between the two groups over 12 months post treatment. The Laser Group showed a small but significant better vision ($p<0.03$) at 12 months compared to the Vitrectomy Group.

The median baseline ETDRS letters read in the PPV group was 58 (range 41-71) (Snellen 20/80, range 20/50 to 20/200). At 6 weeks it was 52 (range 48-68) (Snellen 20/100, range 20/40 to 20/100). At 3 months it was 60 (range 46-70) (Snellen 20/51, range 20/40 to 20/160), whilst at 6 and 12 months it was 60 (range 40-74) (Snellen 20/56, range 20/40 to 20/160) (**see Figure 3.14** and Table 3.3). One patient in the PPV group suffered from MVL at the 12-month final visit

Comparing the vision in the two groups as a *post hoc* analysis using analysis of covariance, there was no significant difference in the vision between the two groups except at 12 months post treatment in which the laser group showed a slight but significant improvement in ETDRS vision compared to the PPV group (p=0.03).

The data were analysed as percentage success rate (defined as either an improvement or stability with no loss of visual acuity greater than 1 line) at each time point compared to baseline. For the laser group it was 88% (6/8) at 6 weeks, 75% at 12 weeks, then 100% at 6 months with a final success percentage at 12 months post laser treatment of 75% (6/8). Similarly for the PPV group success percentage were 57% at 6 weeks, 86% both at 12 and 24 weeks with a final success percentage of 71% at 12 months. This showed that the proportion of patients with successful ETDRS acuity outcome after laser photocoagulation was 4% greater than after PPV.

OCT Results

Following laser treatment foveal thickness was determined at various times post operatively. At baseline the median thickness was 390.5 μm (range 191 to 666 μm) and remained at a similar level (395.5 μm, range 195-636 μm) at 6 weeks post-treatment. During the postoperative period the foveal thickness gradually decreased from the baseline. At 3 months it was 298.5 μm (range 186-424μm), whilst at 6 months it was 318μm (range 184-550 μm). At 12 months it had decreased further to 283μm (range 173-480μm) (**Fig 3.15**). The macular volume and its change over time reflected the changes in the foveal thickness. The median baseline volume in this group was 3.55 mm^3 (range 2.08-5.75 mm^3), which showed a gradual decrease over

the follow up period. At 6 weeks it was 3.48 mm^3 (range 2.33-5.89 mm^3) whilst at 3 months it had decreased to 2.92 mm^3 (range 2.34-4.09 mm^3), with a final decrease to 2.77 mm^3 (range 2.16-4.49 mm^3) at 12 months (**see Figure 3.16**) (Table 3.3).

The median baseline foveal thickness in the PPV group was 364 μm (range 198-607 μm), which did show a gradual decrease over the 12-month post-operative period. At 6 weeks it was 337 μm (range 197-501 μm) with a further decrease at 3 months (median of 342 μm, range 230-424 μm. At 6 months it was 276.5 μm (range 192-501 μm) and by 12 months it had decreased to 337 μm (range 183-467 μm) (**see Figure 3.15**) (**see Figure 3.17** illustrates OCT macula changes seen in the laser group). Similarly, the median baseline volume was 3.23 mm^3 (range 2.26-4.13), which then decreased to 3.04 mm^3 (range 2.34-3.65 mm^3) at 6 weeks, 2.8 mm^3 (range 2.42-3.44 mm^3) at 3 months, 2.46 mm^3 (1.8-3.55 mm^3) at 6 months but at 12 months it had showed a small increase to 2.81 mm^3 (range 2.25-3.97 mm^3) (**see Figure 3.16**) (**see Figure 3.18** illustrates OCT macula changes seen in the vitrectomy group) (Table 3.3)

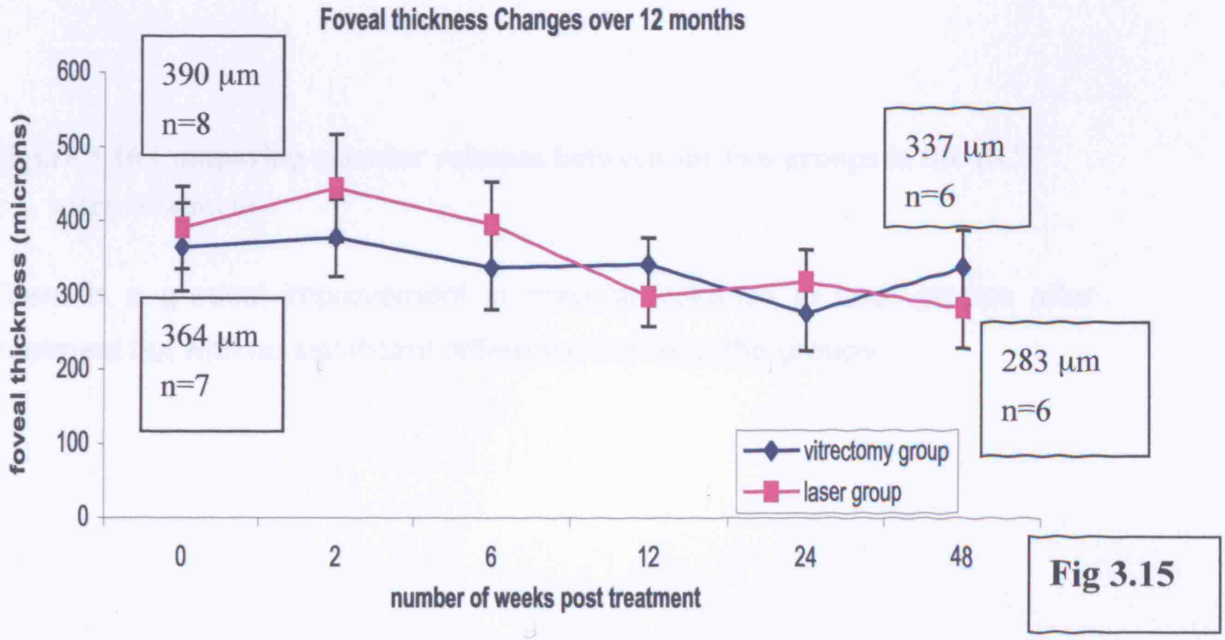


Figure 3.15 Comparing foveal thicknesses between the two groups in the RCT after treatment. There is a gradual improvement in foveal thickness in both groups after treatment but with no significant difference between the

groups.

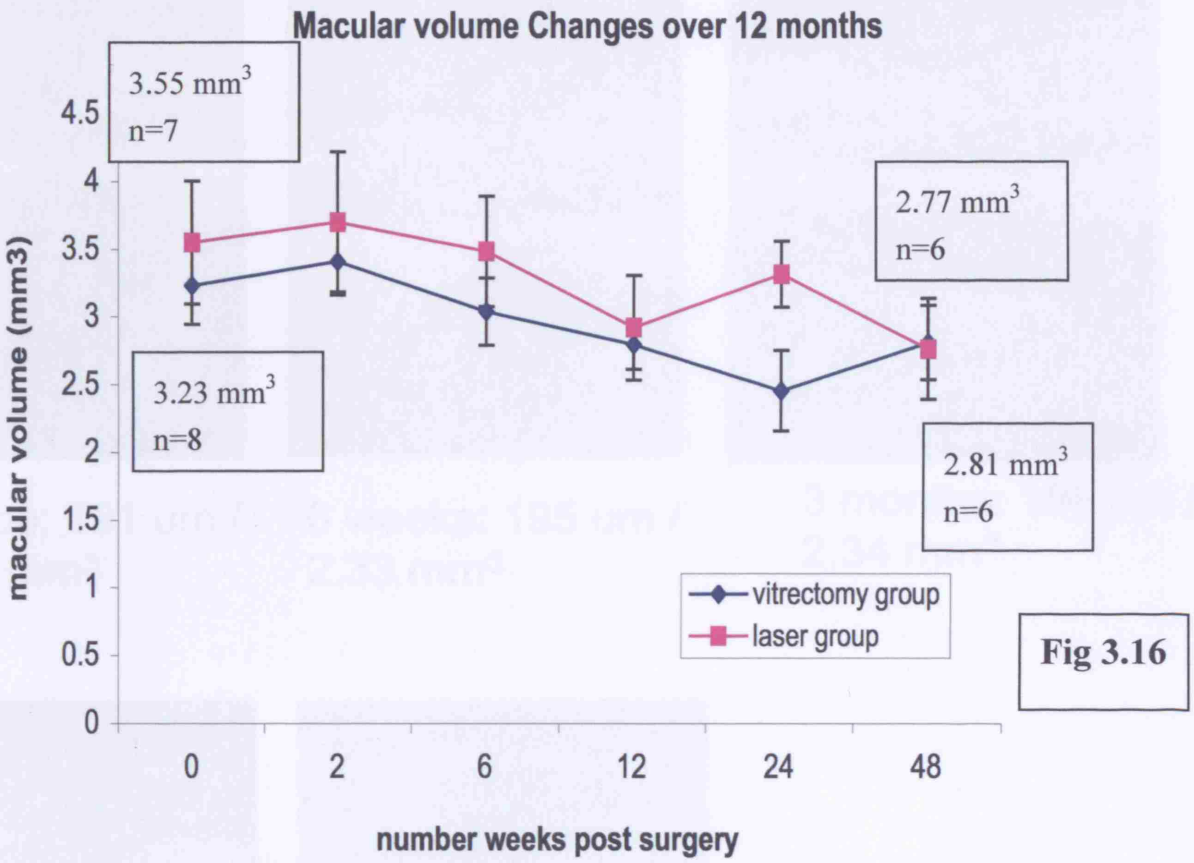
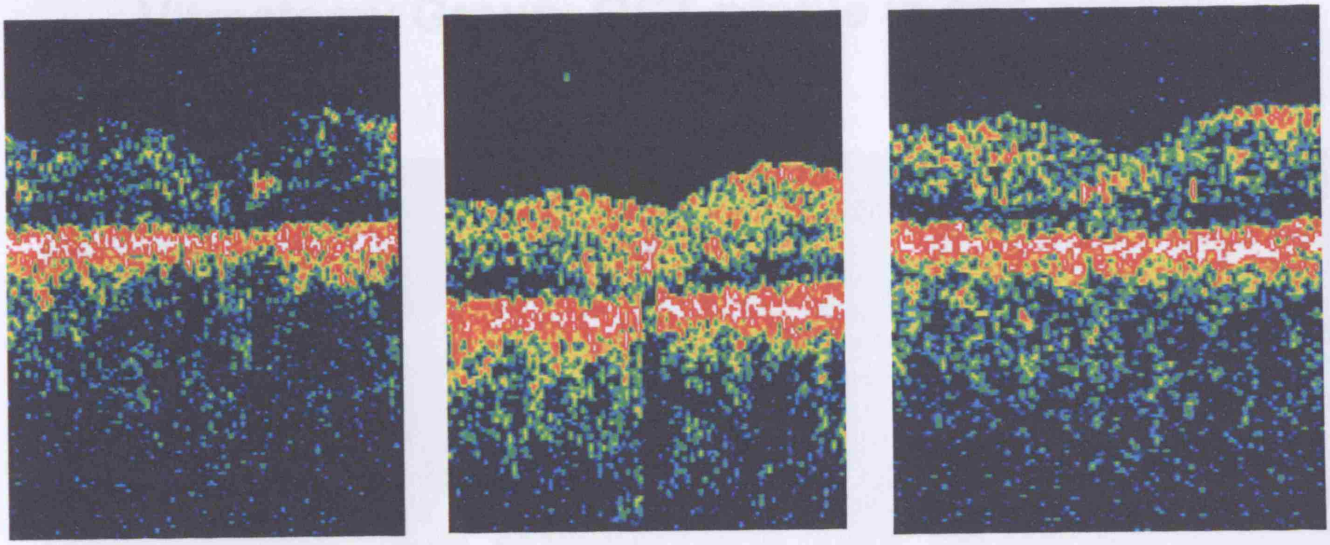


Fig 3.16

Figure 3.16 Comparing macular volumes between the two groups in the RCT after treatment.

There is a gradual improvement in macular volumes in both groups after treatment but with no significant difference between the groups.

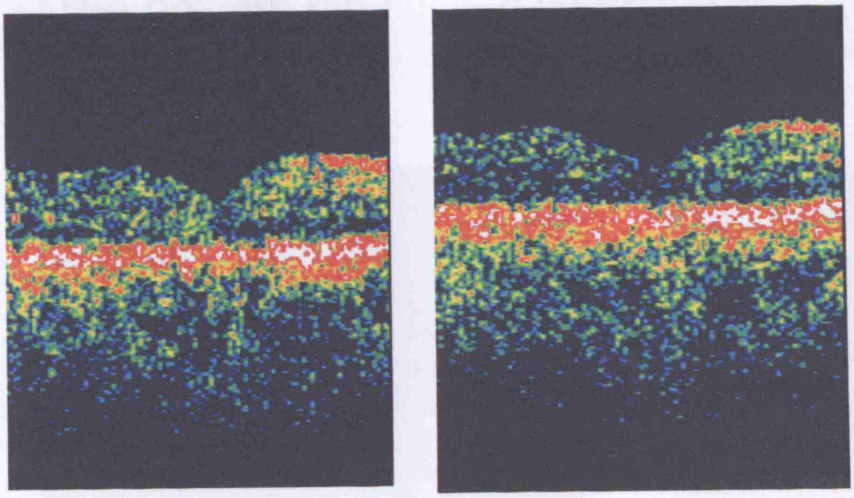
Laser Group: OCT profile in one patient



Pre op: 191 um / 2.08 mm^3

6 weeks: 195 um / 2.33 mm^3

3 months: 186 um / 2.34 mm^3



6 months: 184 um / 2.14 mm^3

12 months: 173 um / 2.16 mm^3

Fig 3.17

Figure 3.17 Macula OCT changes for a patient in the Laser Group after treatment.

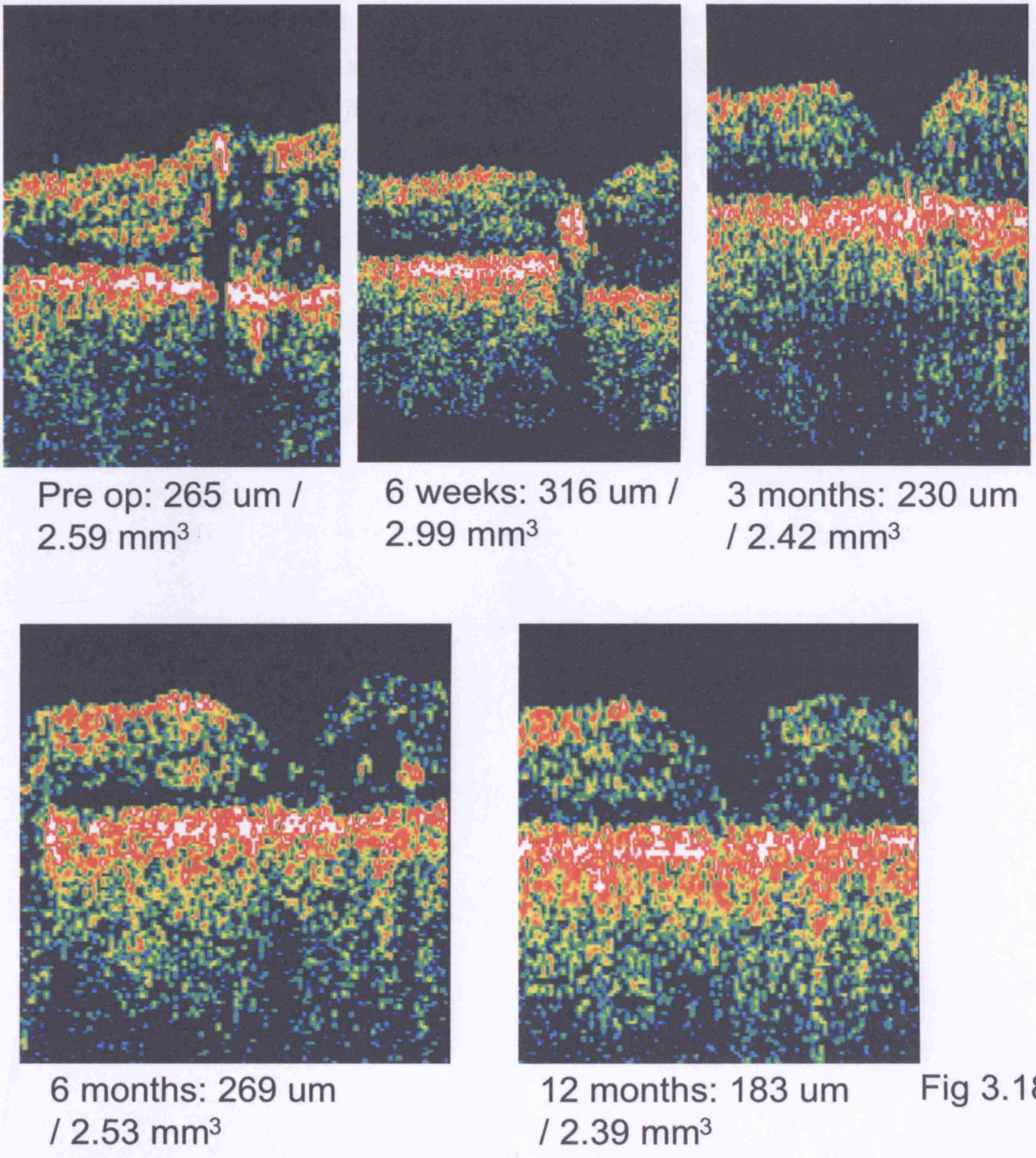


Figure 3.18. Macula OCT changes for a patient in the Vitrectomy Group after treatment

Comparing the changes in ETDRS vision in the two groups over time, the laser group showed a greater level of improvement at each time point compared to the PPV group (**see Figure 3.19**). Similar assessment of changes in the foveal thickness and macular volume confirmed that the laser group showed a greater level of improvement at each follow up visit compared to the PPV group (**see Figure 3.20**). For both graphs each time point is compared to its original baseline level and so measurements which show improvements are positive to the baseline (referenced as the zero line on the graphs).

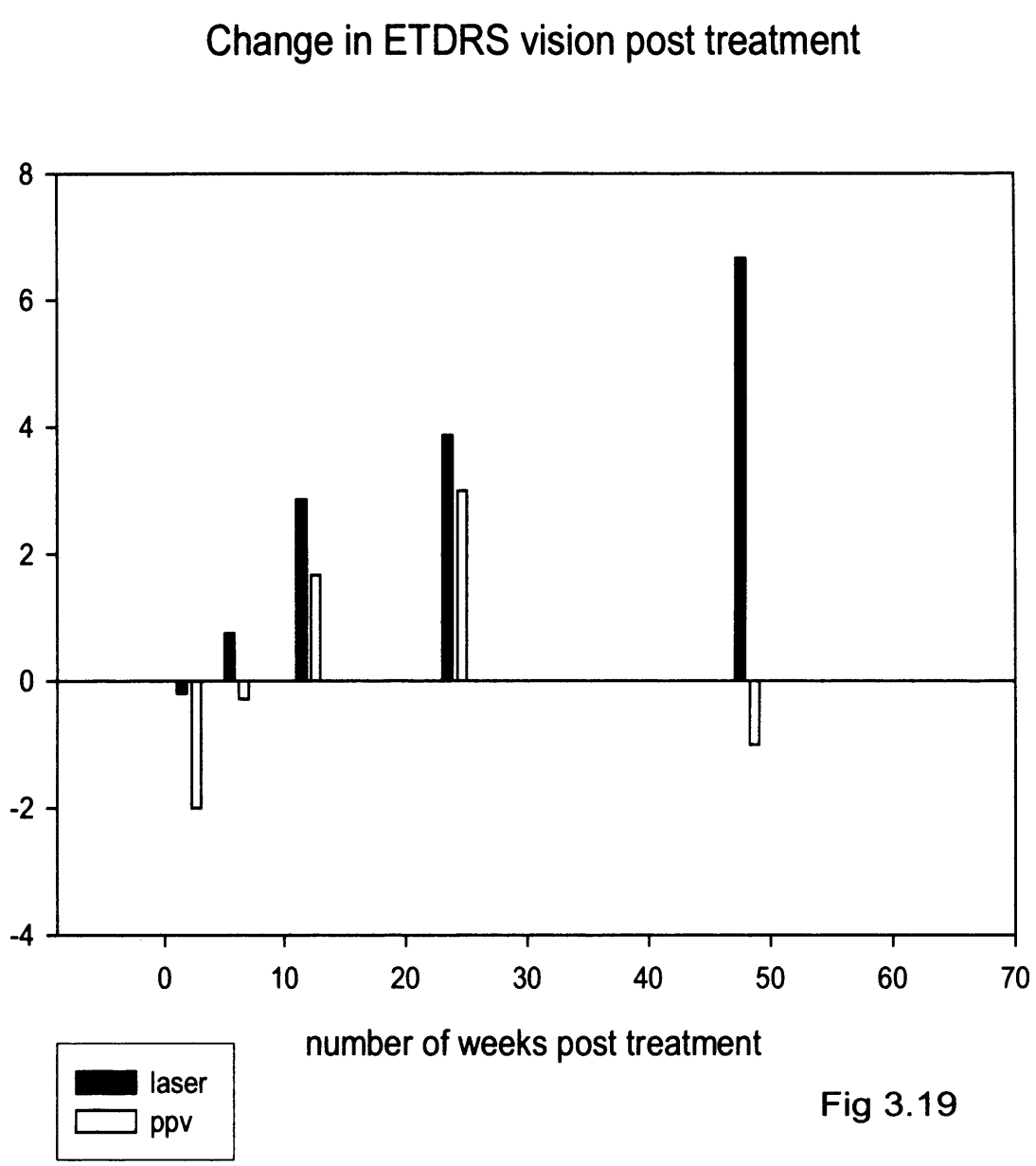
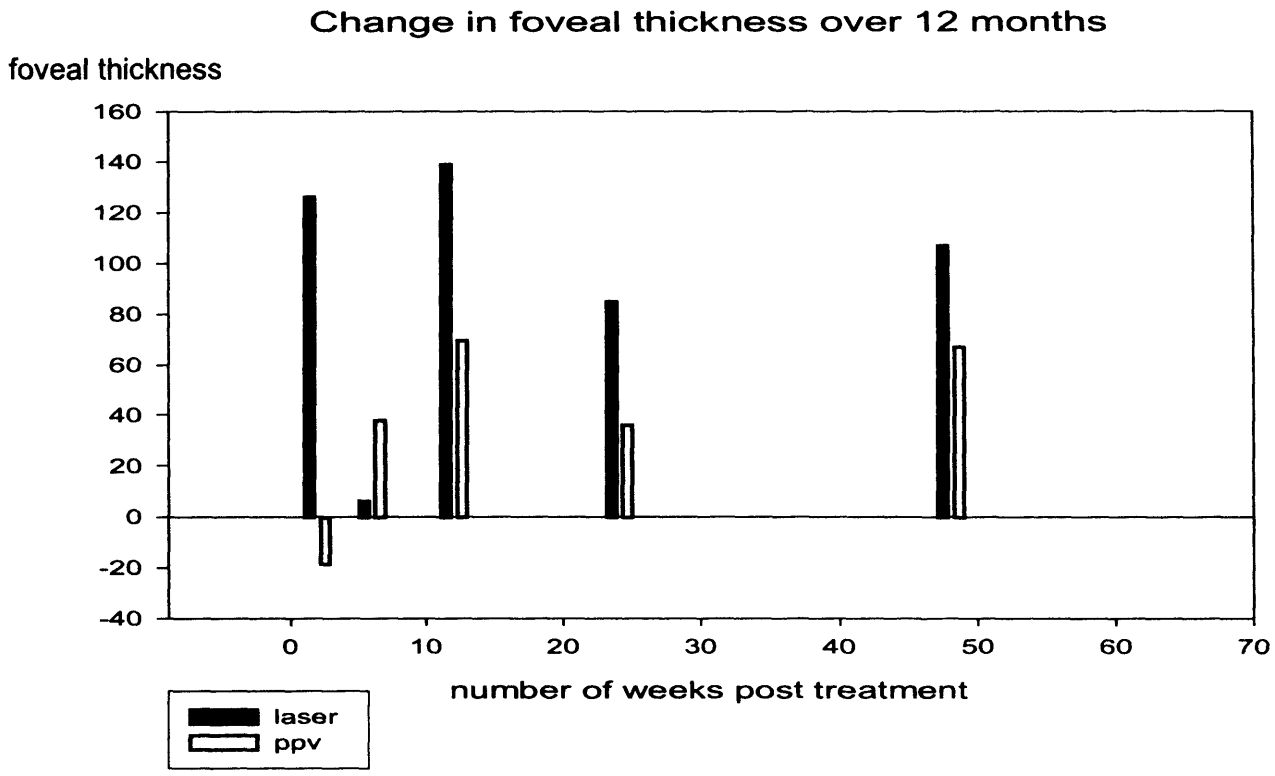


Figure 3.19 Comparing the changes in ETDRS vision between the two groups in the RCT after treatment.

This comparison suggests that the laser group showed a greater improvement than vitrectomy group in terms of EDTRS vision.



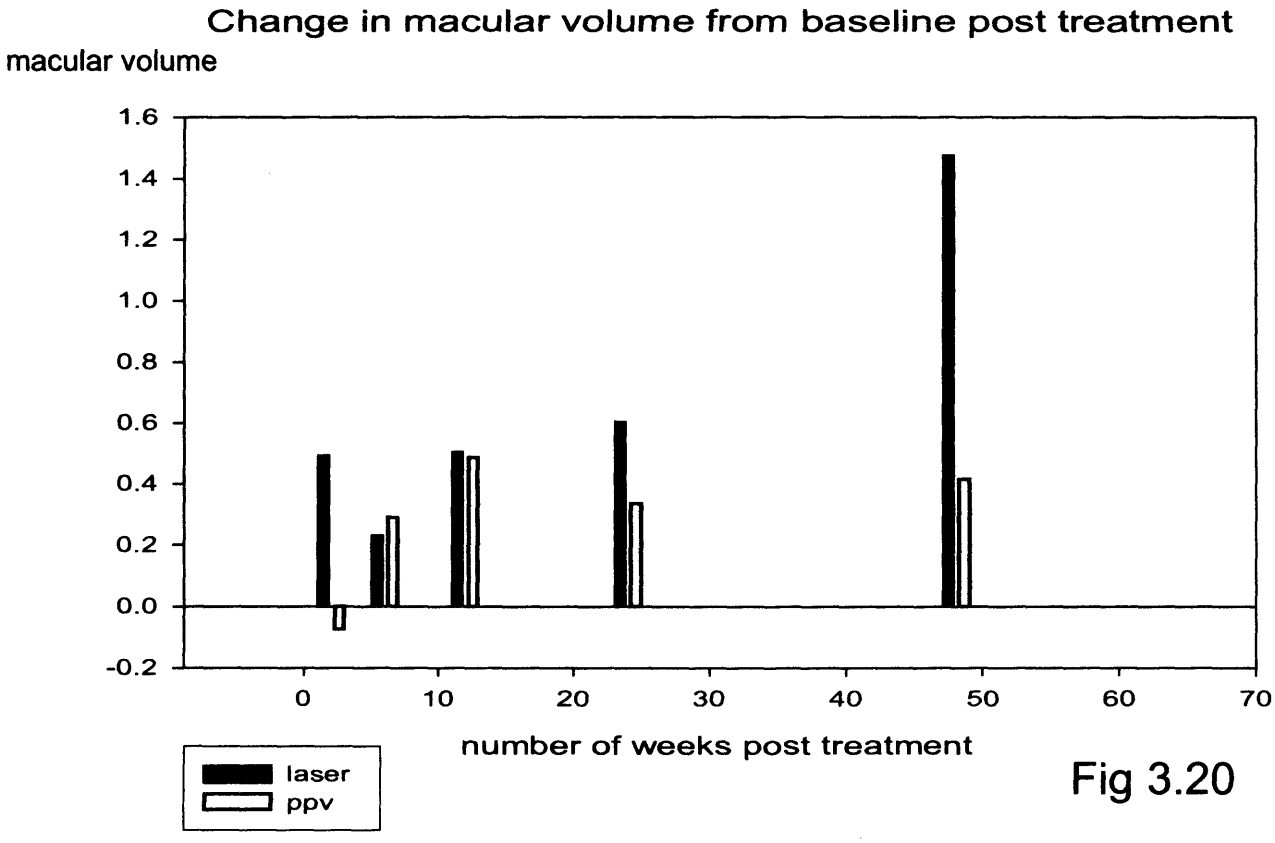


Figure 3.20. Comparing the structural macular changes between the two groups in the RCT after treatment.

This comparison suggests that the laser group showed a greater improvement in the structural parameters than vitrectomy group.

The pre-operative macular profiles on OCT revealed that only 1 patient in each group had a dome-shaped pattern of macular thickening with an attached posterior hyaloid face only at the foveal tip. All the other patients had diffuse-low thickening of the macula with no signs of focal posterior hyaloid separation. The one patient in the PPV group with the dome-shaped profile showed a decreasing foveal thickness and macular volume over the 1 year post operative follow up period so that by 12 months the foveal thickness was 385μm (baseline 472 μm) and the macular volume was 3.18mm^3 (baseline 3.74 mm^3). Reflecting this structural improvement, both the ETDRS vision and the Snellen acuity showed improvements: ETDRS vision improved from 53 letters read at baseline to 63 letters read at 12 months or a baseline corrected Snellen of 20/80 to a 12 months Snellen of 20/50. In the laser patient with dome shaped macular thickening, the foveal thickness remained high despite laser treatment in the early follow up period but by 6 and 12 months post treatment both these structural indices showed improvements. Twelve months foveal thickness was 207 μm (baseline 666 μm) and the macular volume was 2.28 mm^3 (baseline 5.38 mm^3). However the visual acuity remained stable at 20/50. Serial OCT in this patient revealed evidence of spontaneous posterior vitreous separation 3 months after treatment. Two other patients in the laser group had evidence of similar OCT-confirmed spontaneous vitreous detachment during the 1 year follow up period with similar improvement in both the macular structural indices and visual acuity (**see Figure 3.21**).

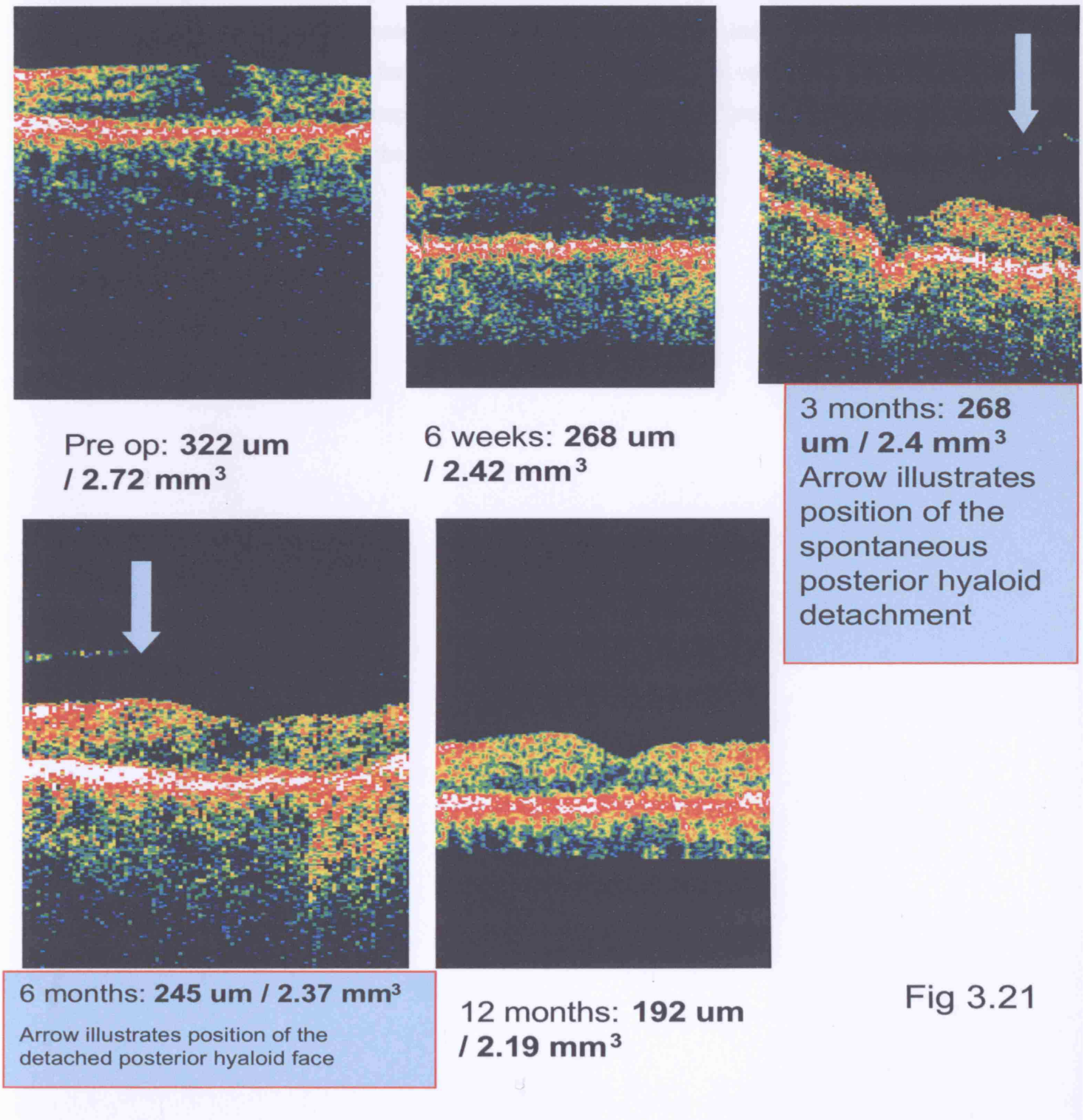


Figure 3.21 OCT captured spontaneous posterior vitreous detachment in a Laser Group patient

FMM Results

The log thresholds of the perifoveal cones showed no significant difference between the two groups after treatment despite both treatments individually showing improvement in the cone function. Comparing the change in cone thresholds over time, the vitrectomy group showed a greater level of perifoveal functional improvement compared to the laser group (**see Figure 3.22**).

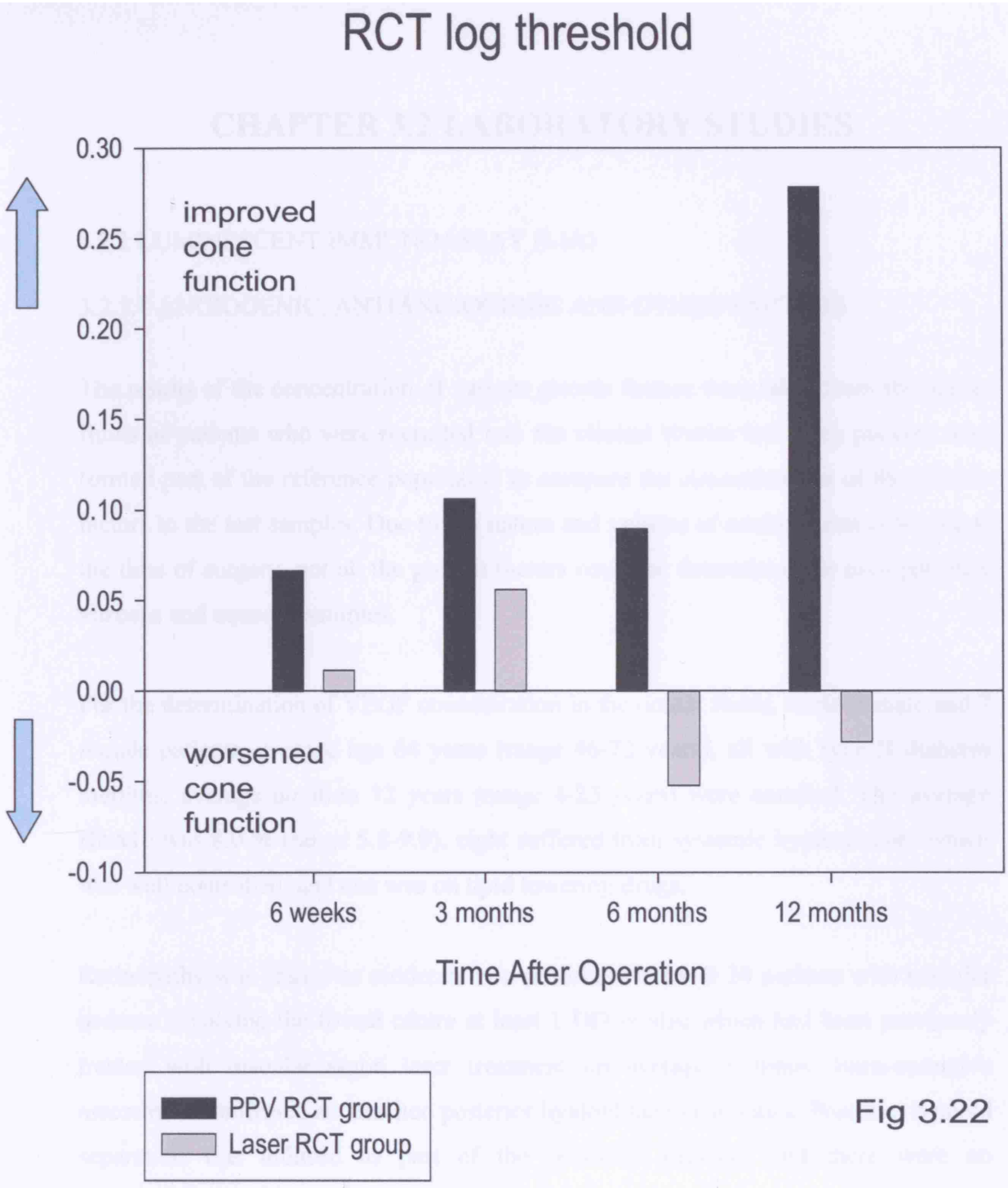


Figure 3.22 Comparing the changes in the cone function between the two groups in the RCT after treatment.

The vitrectomy group showed a greater improvement of perifoveal cone function post treatment

CHAPTER 3.2 LABORATORY STUDIES

3.2.1 LUMINESCENT IMMUNOASSAY (LIA)

3.2.1.1 ANGIOGENIC, ANTIANGIOGENIC AND OTHER FACTORS

The results of the concentration of various growth factors were taken from the ocular fluids of patients who were recruited into the clinical studies including patients who formed part of the reference population to compare the concentrations of the growth factors in the test samples. Due to the nature and volume of ocular fluids collected at the time of surgery, not all the growth factors could be determined for each patient's vitreous and aqueous samples.

For the determination of VEGF concentration in the ocular fluids, thirteen male and 7 female patients, average age 64 years (range 46-72 years), all with type II diabetes mellitus, average duration 12 years (range 4-23 years) were enrolled. The average HbA1c was 8.0 % (range 5.8-9.9), eight suffered from systemic hypertension, which was well controlled, and one was on lipid lowering drugs.

Retinopathy was graded as moderate non-proliferative in all 20 patients with macular oedema involving the foveal centre at least 1 DD in size which had been previously treated with macular argon laser treatment on average 3 times. Intra-operative assessment confirmed an attached posterior hyaloid face in all cases. Posterior hyaloid separation was induced as part of the operative protocol and there were no complications resulting from surgery.

Vitreous samples from eight patients with idiopathic full thickness macular hole and from twenty-two patients with previously lasered proliferative diabetic retinopathy undergoing vitrectomy for either active proliferative or quiescent proliferative - tractional retinal detachment associated with mostly inactive fibrovascular membranes were also collected as refernce samples for comparison of cytokine concentrations.

OCT findings

Two groups were identified based on OCT macular profiles. Group 1 (n=4) had a dome-shaped thickened macula with the posterior hyaloid attached to the apex of the dome corresponding to the fovea (see Figure 23). Group 2 (n=16) had a diffuse low-elevation profile of the thickened macula with no signs of a posterior hyaloid separation (**see Figure 3.2 a**, OCT images of each type of macular profile described).

Baseline growth factor and protein levels

a) VEGF-A

The standard curve of the LIA from which the sample concentrations were determined for VEGF is shown in **Figure 3.23a**)

In the twenty study patients with non-proliferative diabetic retinopathy (NPDR) and clinically significant macular oedema (CSMO) the median vitreous concentration was 957 pg/ml (range 548-2832 pg/ml), whilst it was significantly lower in the macula hole control group (n=8) (239 pg/ml, range 17-380 pg/ml, $p<0.0001$). In the twenty-two patients with PDR the median VEGF concentration was 596 pg/ml (range 88-3213 pg/ml, $p=0.005$ compared to FTMH). The difference between the study group (NPDR) and PDR was also significant ($p=0.006$) (see Table 3.4 and **Figure 3.24**). Of the PDR patients ten had active neovascularization (median 1036 pg/ml, range 765-3213 pg/ml) and twelve quiescent-tractional PDR (median 308 pg/ml, range 88-687 pg/ml) (**Figure 3.24**). Active retinopathy showed significantly greater concentrations of VEGF compared to quiescent disease ($p < 0.001$), and FTMH ($p < 0.001$). Similar concentrations are found in quiescent and FTMH vitreous but NPDR demonstrated a higher concentration than quiescent disease ($p < 0.001$). However there was no difference between active proliferative and NPDR with CSMO ($p > 0.05$) (**see Table 3.6 and Figure 3.24**).

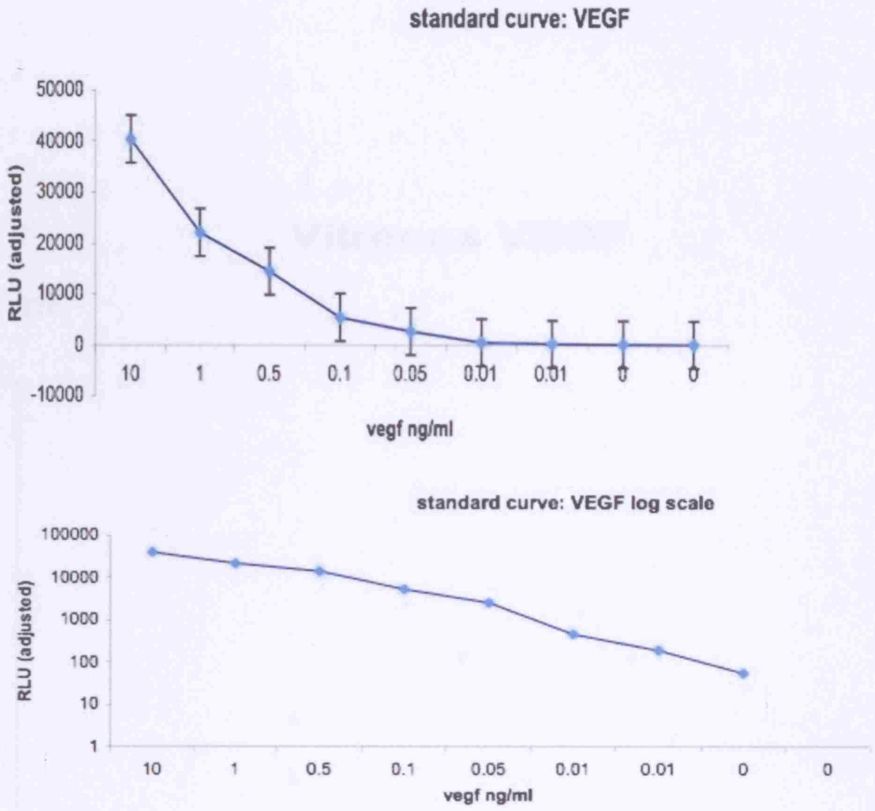


Fig 3.23a

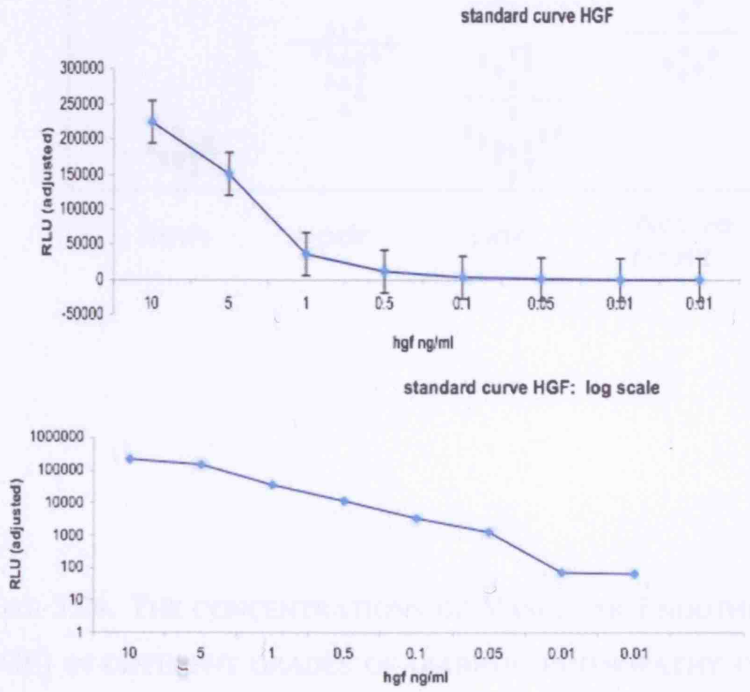


Fig 3.23b

Fig 3.23 a/b. Standard curves for VEGF and HGF respectively

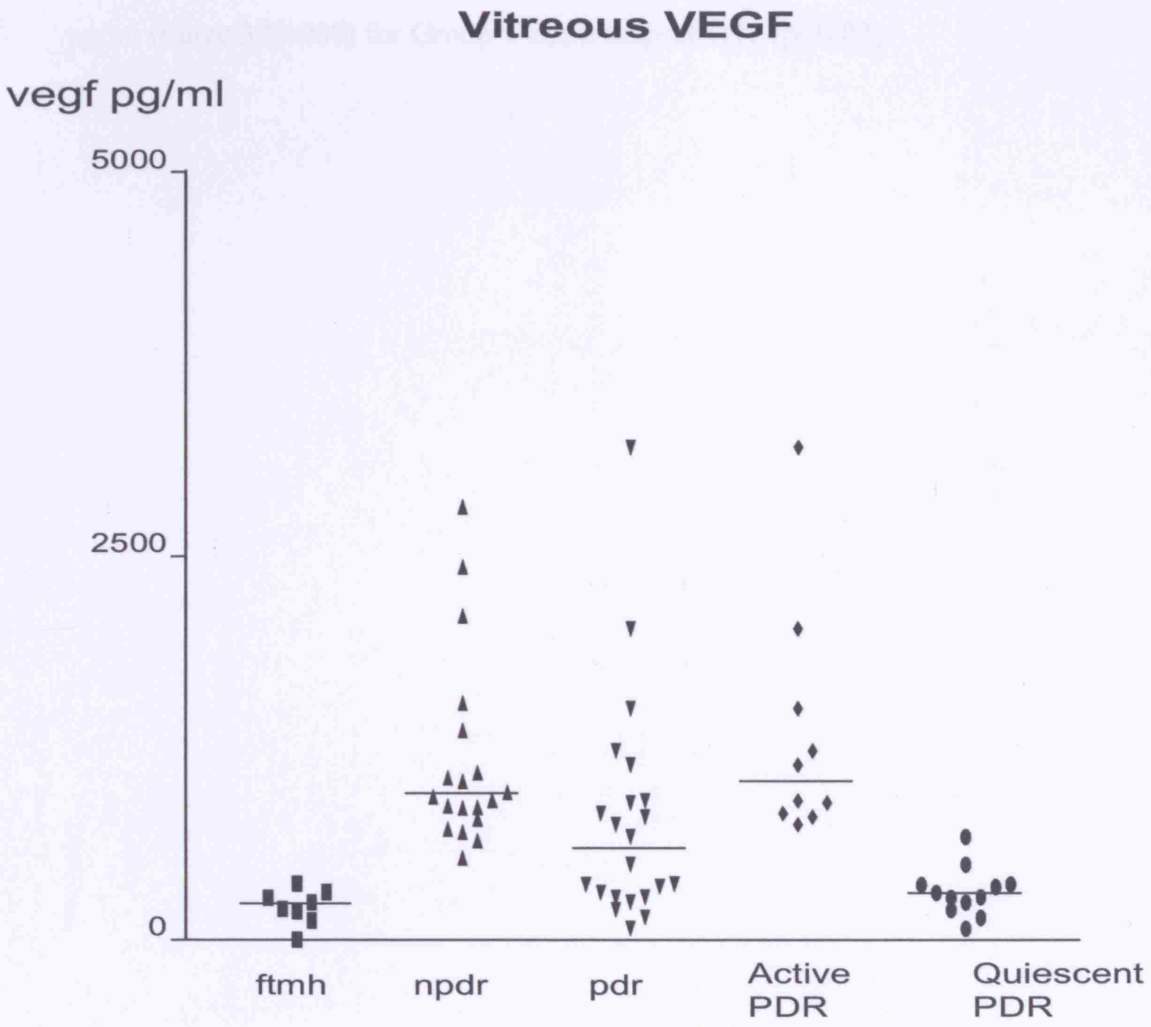
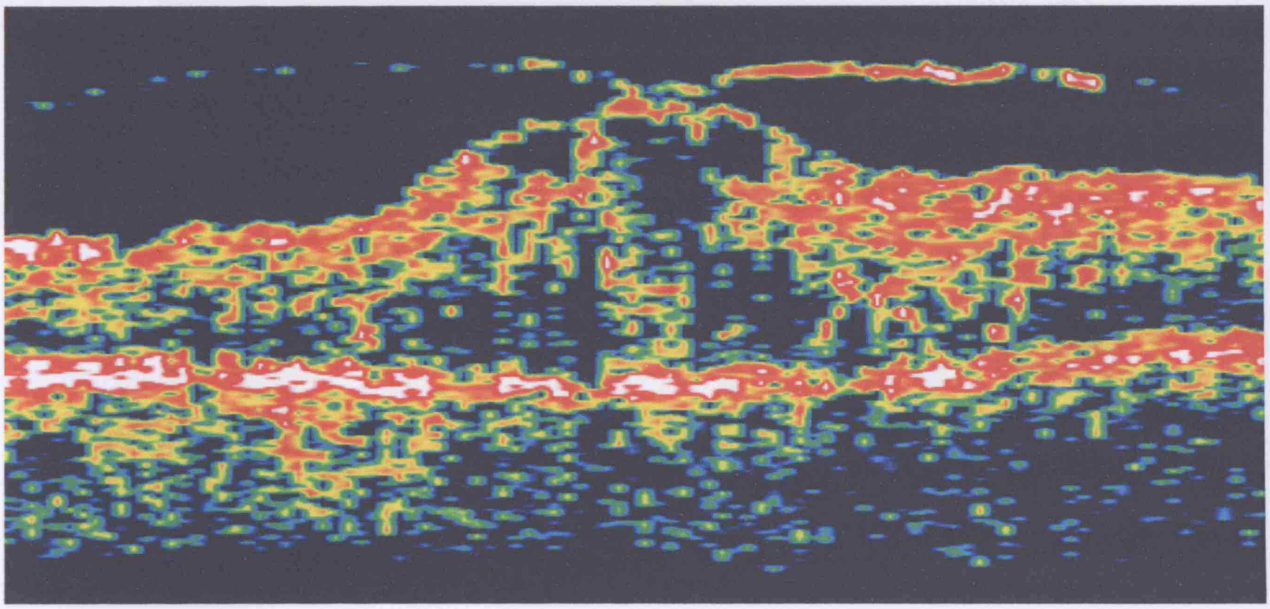


Fig 3.24

FIGURE 3.24. THE CONCENTRATIONS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN DIFFERENT GRADES OF DIABETIC RETINOPATHY COMPARED TO REFERNCE GROUPS. (See Table 3.6 for reference for significant values compared to FTMH) (FTMH= full thickness macular hole, NPDR= non proliferative diabetic retinopathy, PDR= proliferative diabetic retinopathy, qPDR= quiescent PDR, aPDR= active PDR)

The VEGF-A (VEGF) concentrations in the vitreous and aqueous at baseline were different between the two macular profiles. Median vitreous VEGF concentration was 806 pg/ml (range 548-877 pg/ml) in Group 1 (dome-shaped) **(Figure 3.25a)** and 1059 pg/ml (range 715-2832) in Group 2 (diffuse-low elevation) ($p=0.04$) **(Figure 3.25b)**. Median aqueous VEGF concentrations were 207 pg/ml (range 145-353) and 415 pg/ml (range 320-960) for Group 1 and 2 respectively ($p<0.02$).



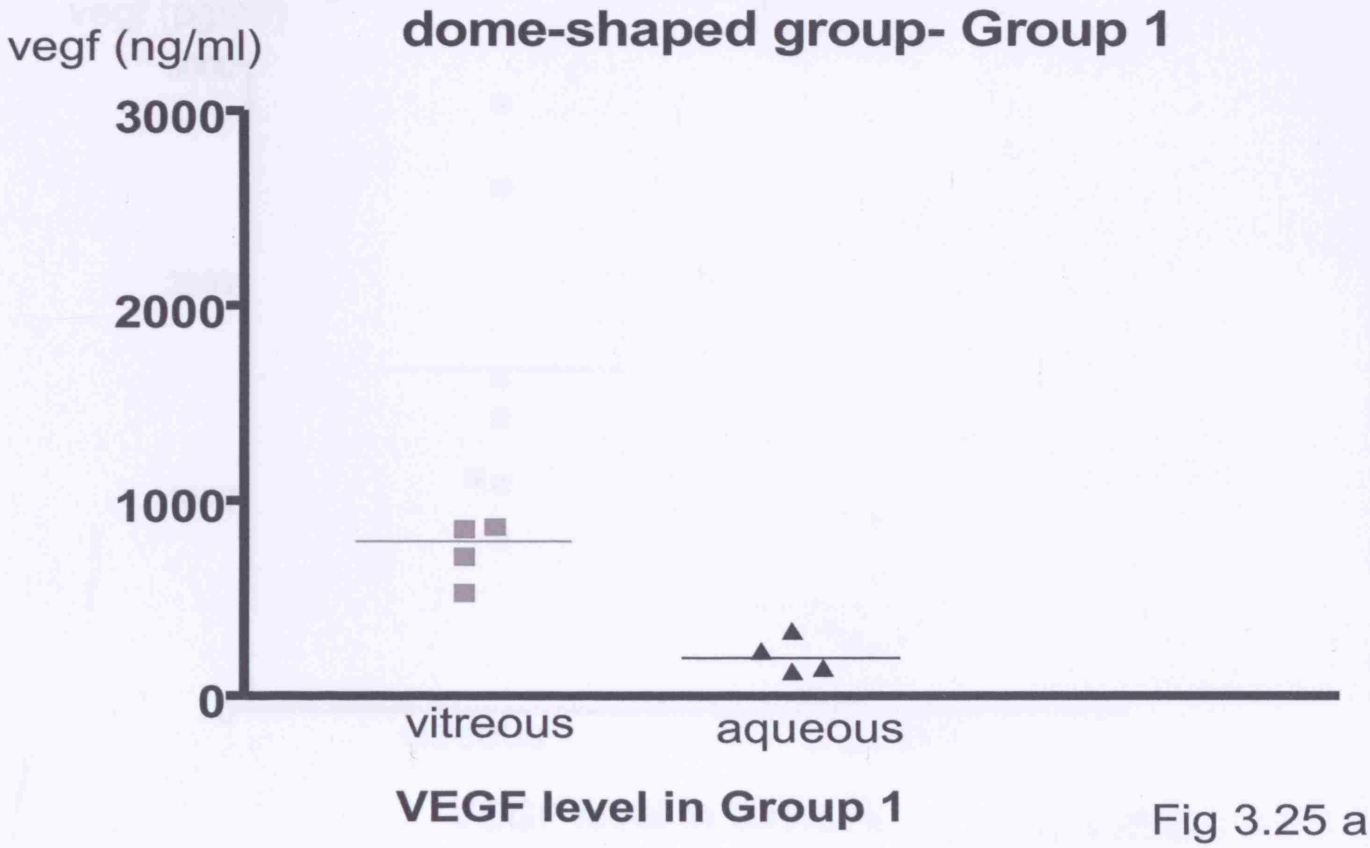
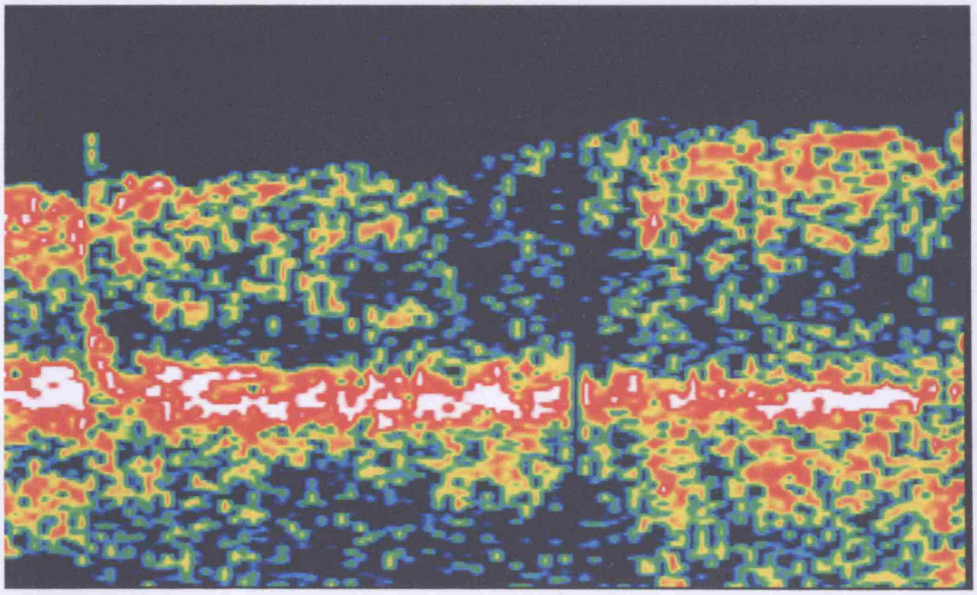


Fig 3.25 a

Fig 3.25a. Dome-shaped macular profile and corresponding ocular fluid concentration.



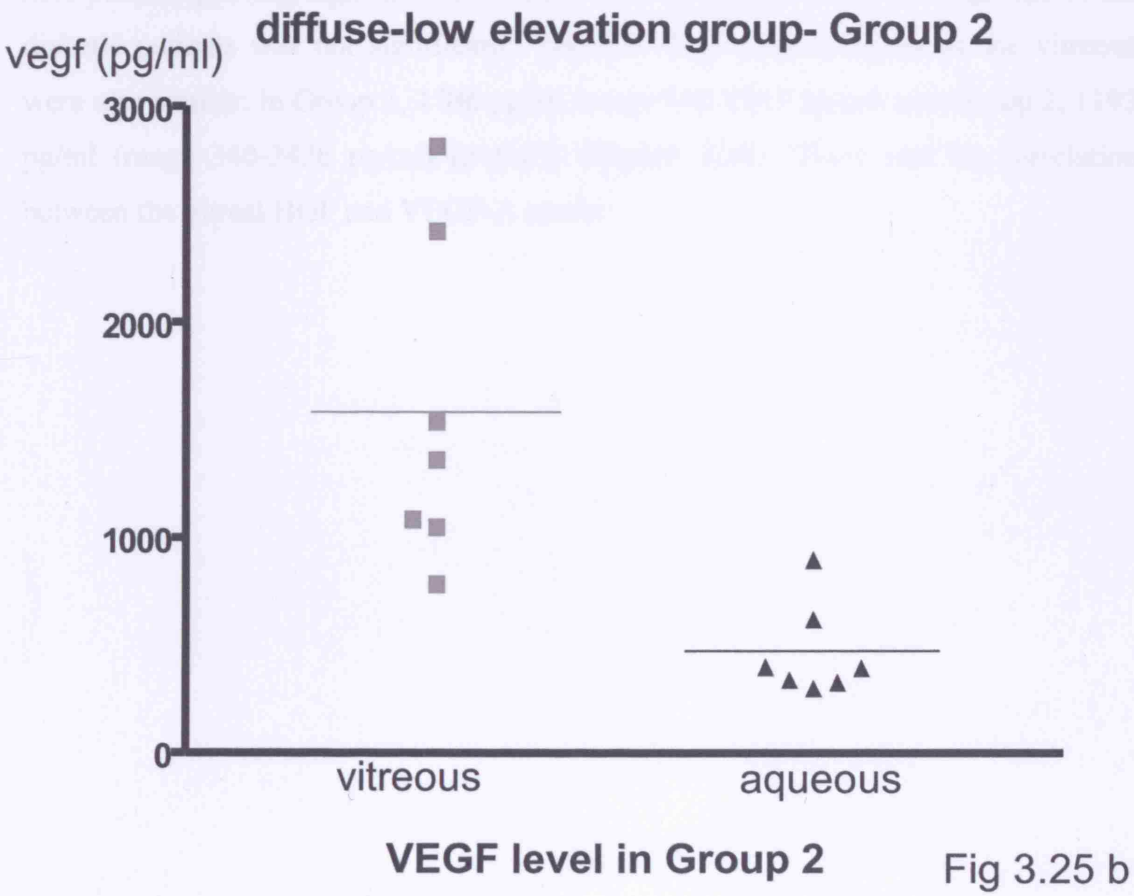


Fig 3.25b. Dome-shaped macular profile and corresponding ocular fluid concentration.

Vitreous and aqueous VEGF concentrations between these two macular profiles were significantly different ($p=0.04$ and $p<0.02$ respectively).

b) HGF (see Table 3.4)

The standard curve of the LIA from which the sample concentrations were determined for HGF is shown in **Figure 3.23b**.

HGF concentrations in the diabetic patients were raised. The study group (NPDR) had a median concentration of 1500 pg/ml (range 361-2542 pg/ml) with PDR concentration at 2675 pg/ml (range 307-3707) compared to FTMH (416 pg/ml, range 381-750 pg/ml) with these differences in the concentration compared to the macula hole patients proving significant ($p< 0.02$). The difference between the groups of the diabetic patients was not significant. Median HGF concentrations in the vitreous were also similar: in Group 1, 1786 pg/ml (range 992-2543 pg/ml) and Group 2, 1193 pg/ml (range 340-2426 pg/ml) ($p>0.05$) (**Figure 3.26**). There was no correlation between the vitreal HGF and VEGF-A levels.

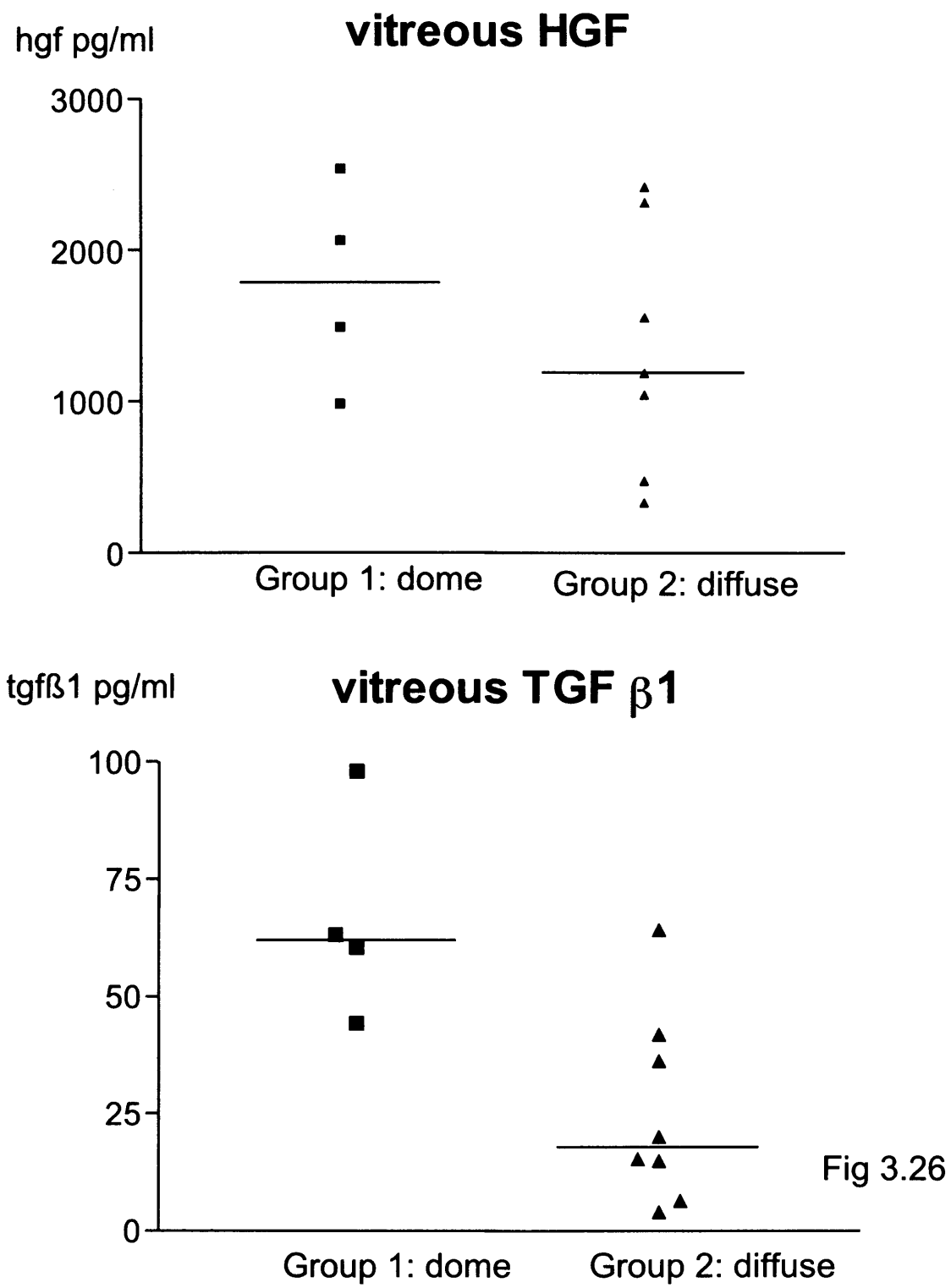


Fig 3.26

Figure 3.26. The concentrations of (a) HGF and (b) TGF β in the vitreous between the two macular profile groups.

There was no difference in the HGF concentration between the two macular profile groups, but there was a significant difference in the TGF β concentration between the two groups, greater in Group 1 than in Group 2 (p=0.03).

c) TGF-β1 (see Table 3.4)

The standard curve of the LIA from which the sample concentrations were determined for both TGF-β1 and MMP 9 (**Figure 3.27a and b respectively)**

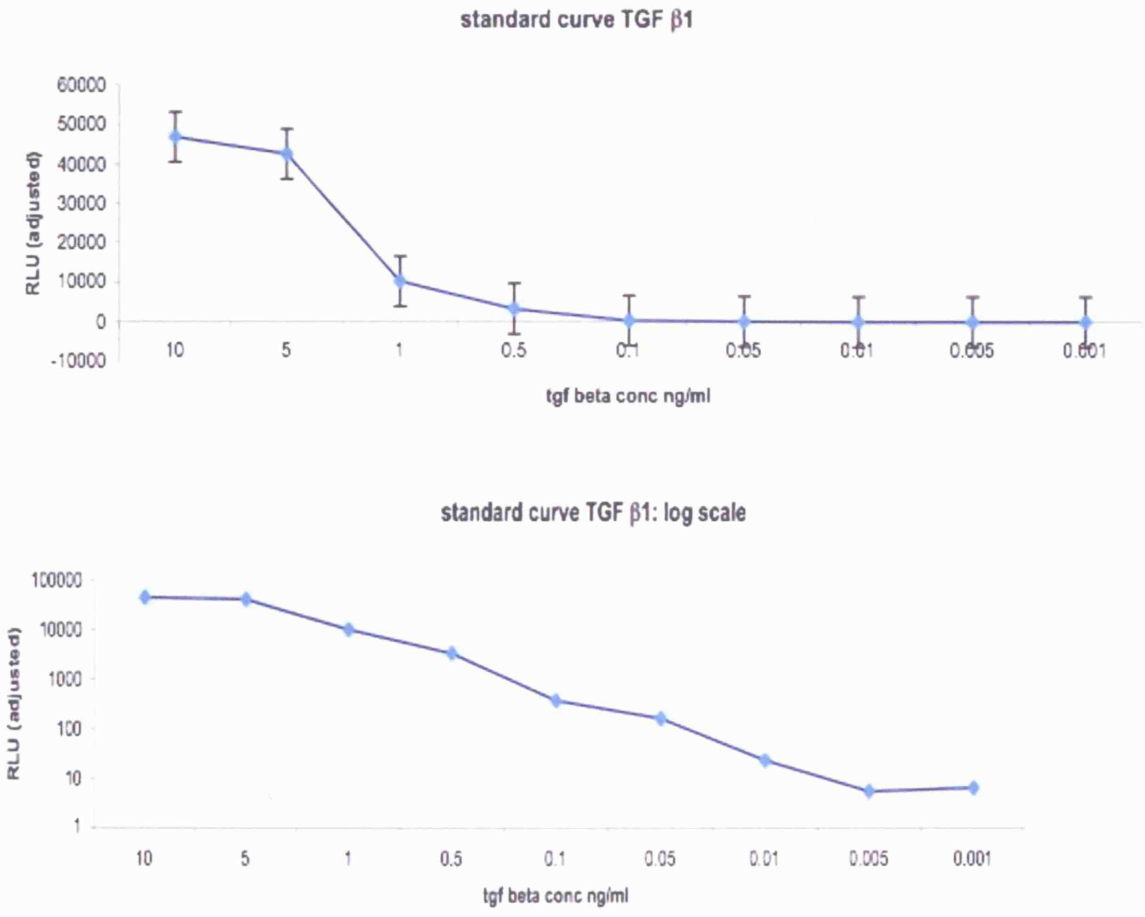


Fig 3.27a

Figure 3.27a: Standard curve for TGF β1. Sample concentrations were determined from this curve.

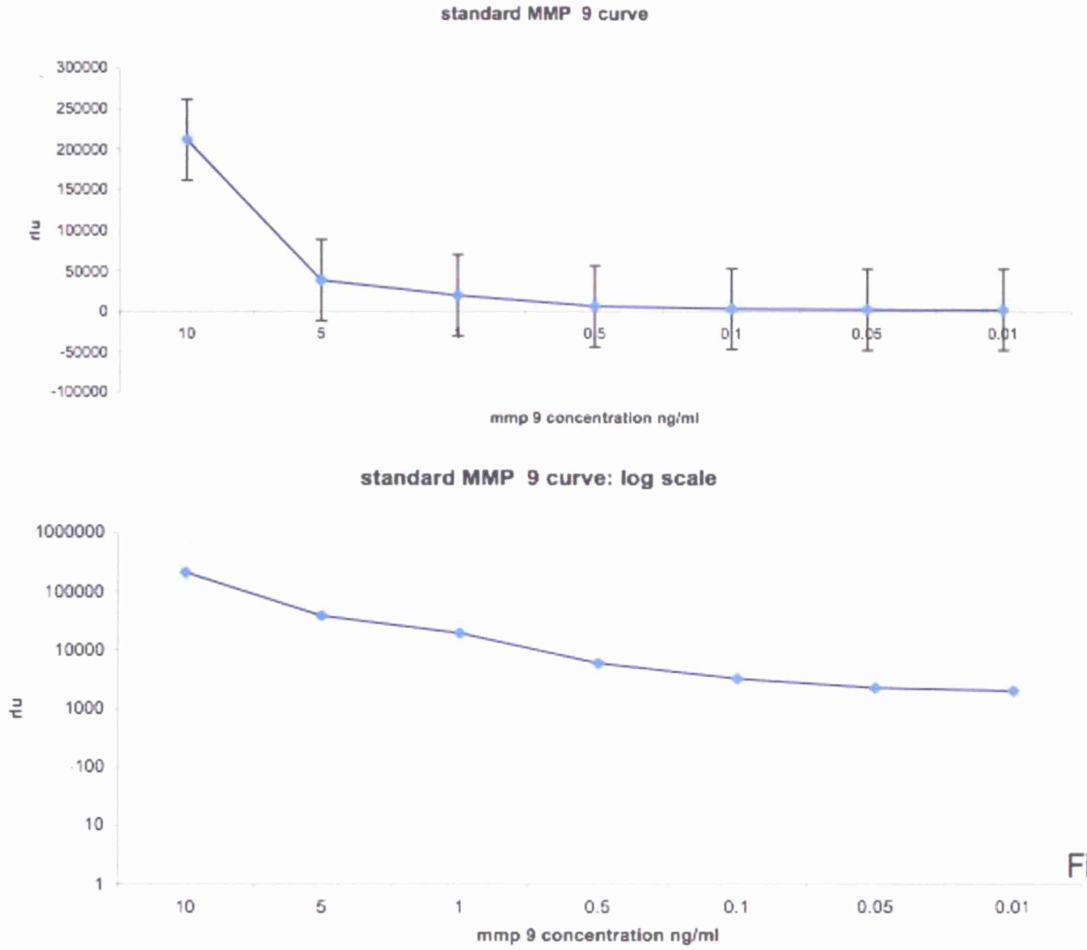


Fig 3.27b

Figure 3.27 b: Standard curve for MMP 9. Sample concentrations were determined from this curve.

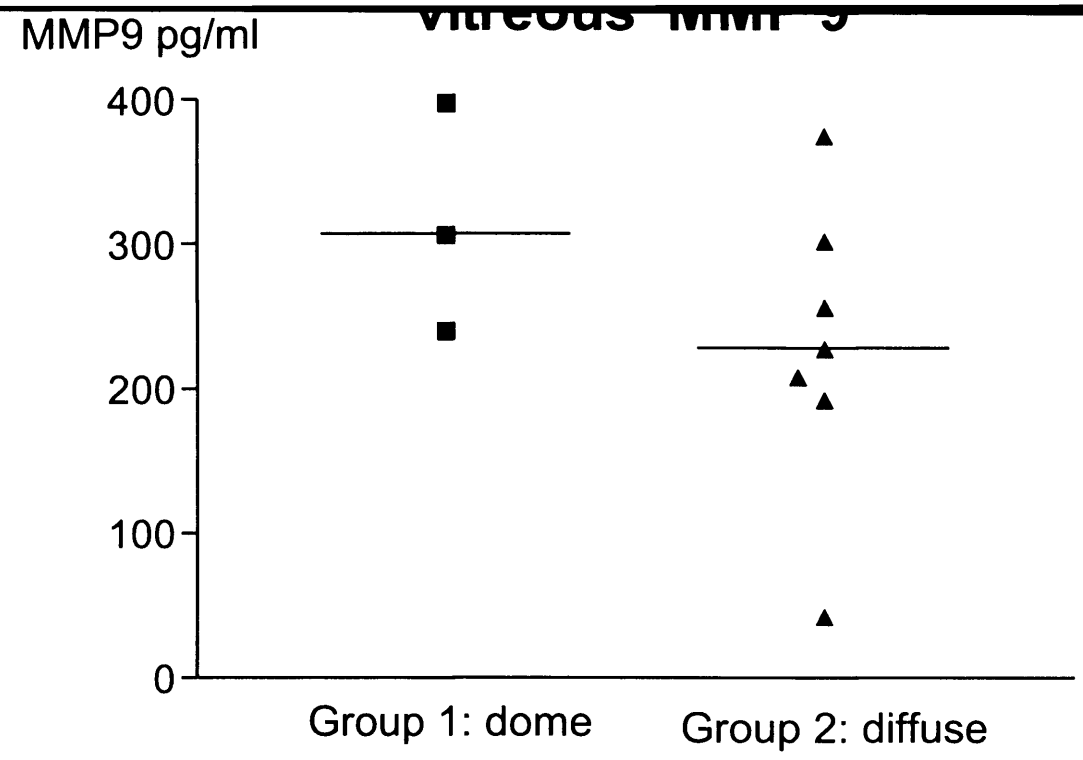
The median vitreous TGF β1 concentration in the study patients was 23 pg/ml (range 9-98 pg/ml) compared to FTMH (927 pg/ml, range 370-1183 pg/ml) and PDR 213 (range 100-403 pg/ml). The differences between the study NPDR and PDR patients ($p<0.01$) and between NPDR and FTMH patients ($p<0.001$) were significant. The TGF β1 concentration in the vitreous was also different between the two groups: the concentration being raised in Group 1 (44 pg/ml, range 44-98 pg/ml) compared to Group 2 (18 pg/ml, range 4-64 pg/ml) ($p=0.03$) (**Figure 3.26**).

f) MMP 9 (Table 3.4)

The median concentrations of vitreous MMP 9 were greater than that found in the control patients (Table 3.4) and also showed no significant difference between the two groups (**Figure 3.28**)

Protein Concentration

There were significant differences in the concentration of protein in the vitreous with a lower median concentration in Group 1 (0.5 +/- 0.12 mg/ml) compared to Group 2 (4 +/- 3 mg/ml) (p=0.04) (**Figure 3.28**).



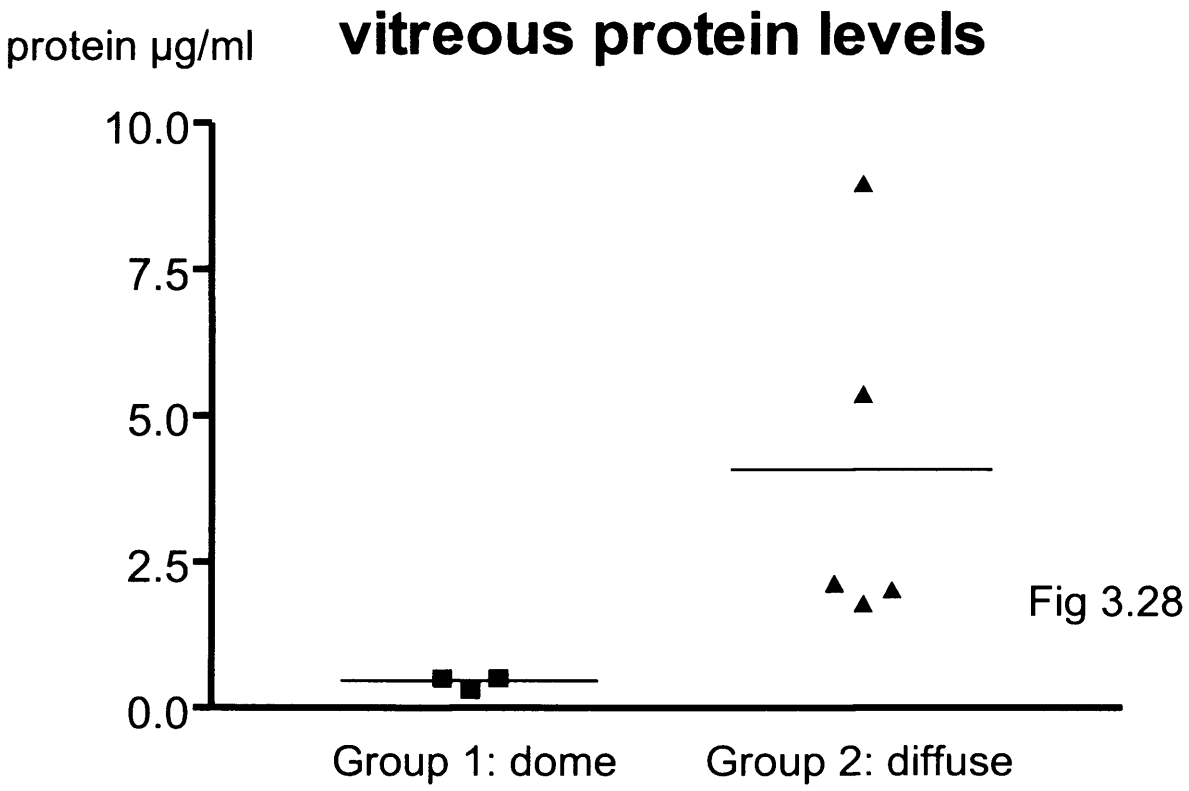


Fig 3.28

Figure 3.28: The concentrations of (a) MMP 9 and (b) protein in the vitreous between the two macular profile groups.

There was no difference in the MMP 9 concentration between the two macular profile groups. There was a small, but significant difference in the protein concentration between them (p=0.03) with Group 1 having the lower concentration compared to Group 2.

c) Soluble Flt 1 Receptor (see Table 3.4)

The standard curve ranged from 100 pg/ml to 100 ng/ml with a sensitivity of 100 pg/ml and all samples fell within this range (interassay CoV was 20% whilst the intra CoV was 7.5%) (**Figure 3.29a**).

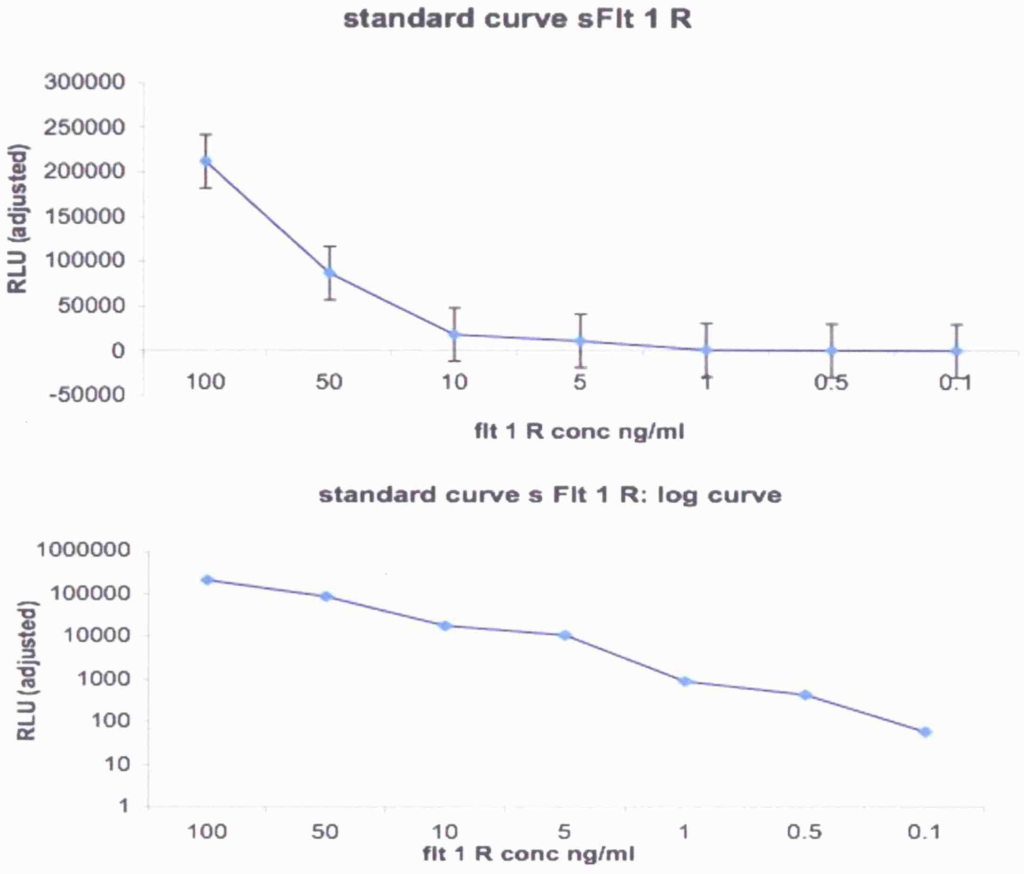


Fig 3.29a

Figure 3.29a: Standard curve for soluble Flt 1 receptor.

Sample concentrations were determined from this curve.

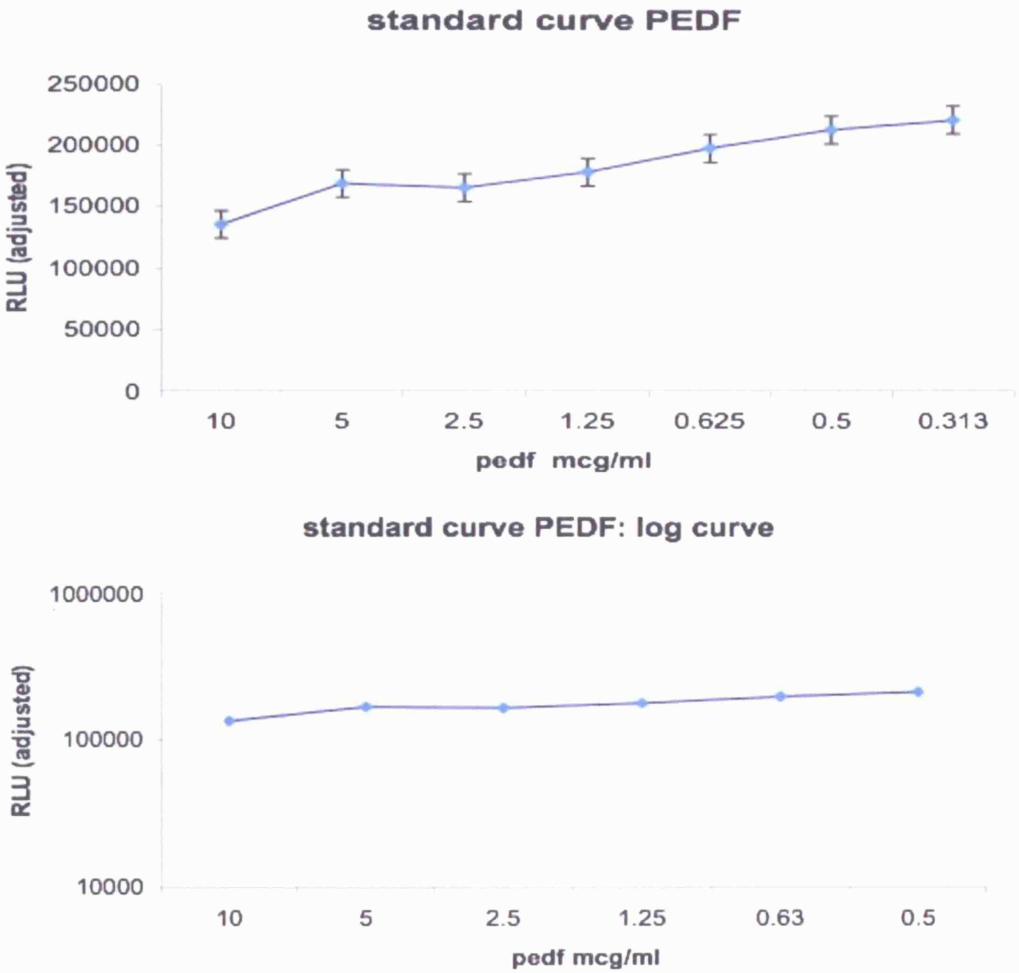


Fig 3.29b

Figure 3.29 b: Standard curve for PEDF.

Sample concentrations were determined from this curve.

In the diabetic vitreous the median concentration of soluble Flt 1 was lower (45 ng/ml, range 20-70 ng/ml) compared to macular hole (73 ng/ml, range 66-77 ng/ml) ($p<0.0001$). In NPDR (study group) the concentration was 49 ng/ml (range 42-70 ng/ml) and in PDR it was 42 ng/ml (range 20-45 ng/ml). The macular hole

concentration was significantly higher compared to NPDR (p=0.0001) and PDR (p=0.012). However there was no difference in the median concentration between the two diabetic states (p=0.18) (**Figure 3.30**).

d) PEDF (see Table 3.4)

The standard curve for PEDF ranged from 0.3 μg/ml to 10 μg/ml and all values of samples fell within this range (**Figure 3.29b**).

Overall in diabetic eyes, the median vitreous Pigment Epithelium-Derived Factor was 1.36 μg/ml (range 0.68-7 μg/ml) compared to 2.6 μg/ml (range 1.5-6.6 μg/ml) in eyes with idiopathic macular hole ($p<0.05$). In the study eyes with non-proliferative retinopathy with fovea-involving CSMO (NPDR-CSMO) it was 1.59 μg/ml (range 0.68-7 μg/ml) whilst it was significantly lower in eyes with proliferative diabetic retinopathy (the median concentration was 1.27 μg/ml, range 0.6-1.7 p=0.02) (**Figure 3.30**). There was no difference between macular hole and non-proliferative retinopathy (p=0.2), but the concentrations for macular hole patients were significantly higher than for proliferative retinopathy ($p< 0.009$).

Anti angiogenic growth factors

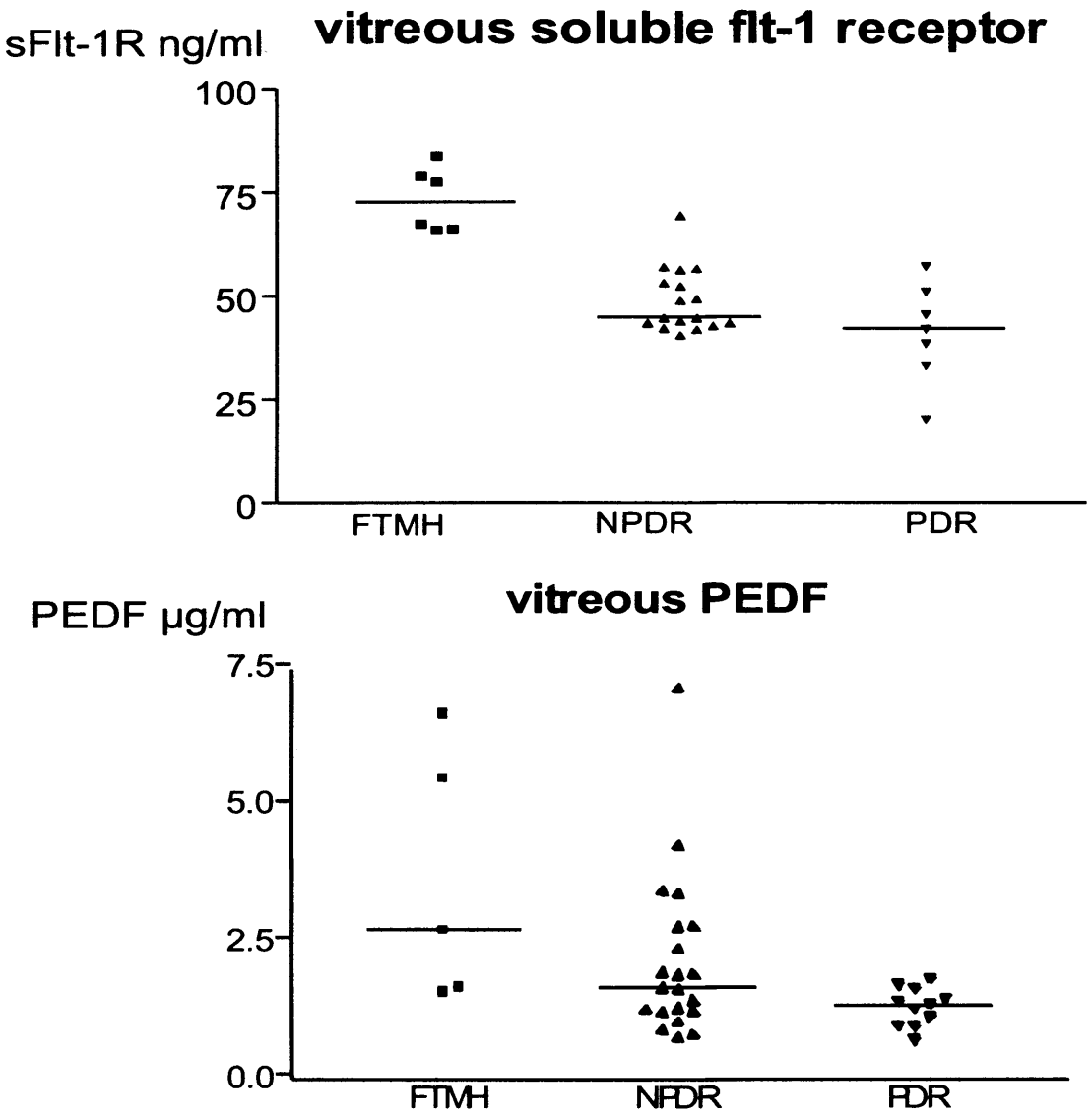


Fig 3.30

Figure 3.30: The concentration of anti-angiogenic growth factors (soluble flt-1 Receptor and PEDF) in the vitreous of diabetic and Reference patients.

In the diabetic vitreous the median concentration of soluble Flt 1 was significantly lower compared to FTMH ($p<0.0001$). The FTMH concentration was significantly higher compared to NPDR (p-0.0001) and PDR ($p=0.012$). Similarly overall in the diabetic eyes, the median vitreous PEDF was significantly lower compared to FTMH ($p=0.05$). However the concentration in FTMH patients were significantly higher than for proliferative retinopathy ($p< 0.009$).

Table 3.6 Summary of the median growth factor concentrations in the vitreous in the different diabetic clinical states (for full ranges see text) (ND= not determined: * denotes significantly different $p < 0.05$ compared to control macular hole).

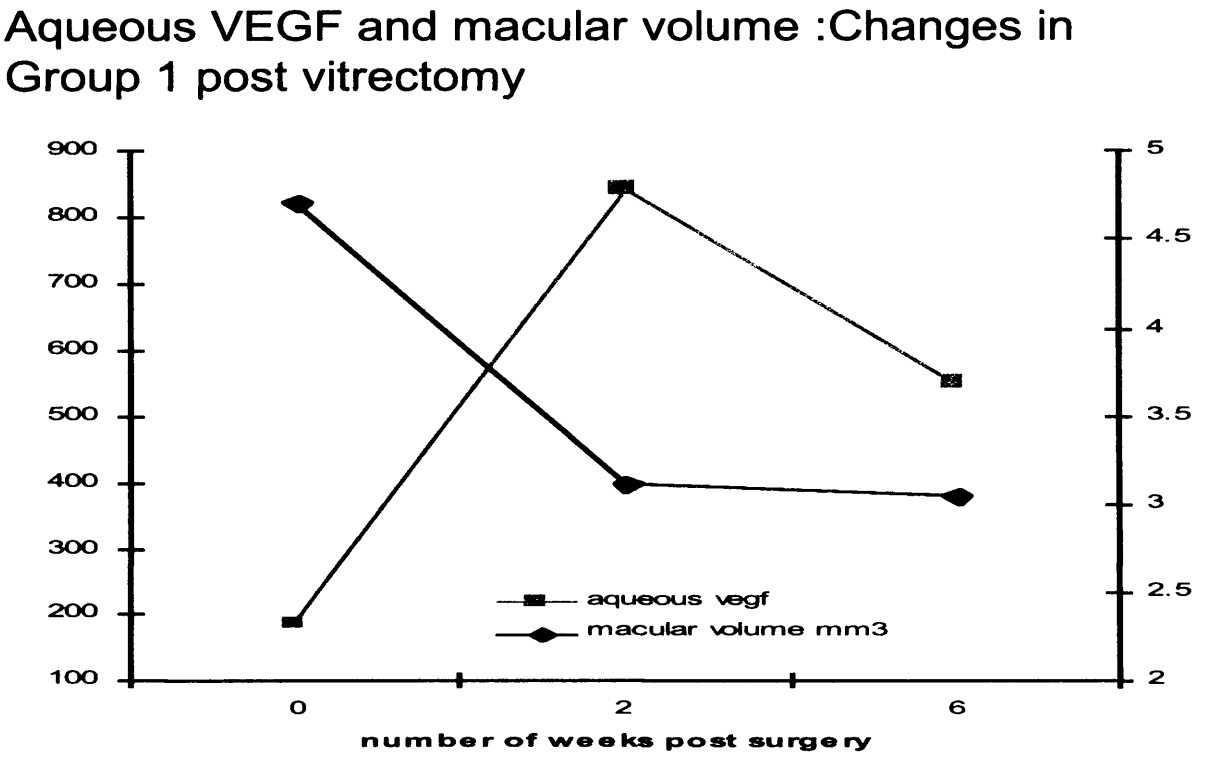
	Control macular hole (FTMH)	Diabetic vitreous	PDR	Active PDR	Quiescent PDR	NPDR (study patients) with CSMO	Group 1 OCT macular profile	Group 2OCT macular profile
VEGF-A (pg/ml)	239	ND	596 *	1036*	308	957 *	806	1059
TGF β1 (pg/ml)	927	ND	213	ND	ND	23 *	44	18
HGF (pg/ml)	416	ND	2675*	ND	ND	1500*	1786	1193
MMP 9 (pg/ml)	83	ND	286	ND	ND	279	307	228
PEDF (μg/ml)	2.6	1.36 *	1.27 *	ND	ND	1.59	ND	ND
Sol Flt 1 Receptor (ng/ml)	73	45 *	42 *	ND	ND	49 *	ND	ND

Table 3.7 Summary of aqueous VEGF in both OCT-Groups.

	Group 1 (dome-shaped) (pg/ml)	**Group 2 (diffuse-elevated) (pg/ml)**
baseline	187	512
2 weeks	843	617
6 weeks	549	228

Post operative macular volume and aqueous VEGF changes

In Group 1, the macular volume decreased by an average of 1.21 mm^3 at 6 weeks whilst the aqueous VEGF increased from a median of 187 pg/ml to a median of 549 pg/ml (194 % increase) (**Figure 3.31**). In Group 2 the macular volume decreased by an average of 0.37 mm^3 at 6 weeks in tandem with the aqueous VEGF, which decreased from a median of 512 pg/ml to a median of 229 pg/ml (a 55 % decrease) (**Figure 3.31**). There was a significant improvement at 6 weeks in the macular volume in the diffuse-low elevation group (Group 2) ($p<0.04$) whilst no such significant improvements were seen in the dome-shaped group (Group 1) (Table 3.7). There was only sufficient post operative aqueous available to determine VEGF concentration.



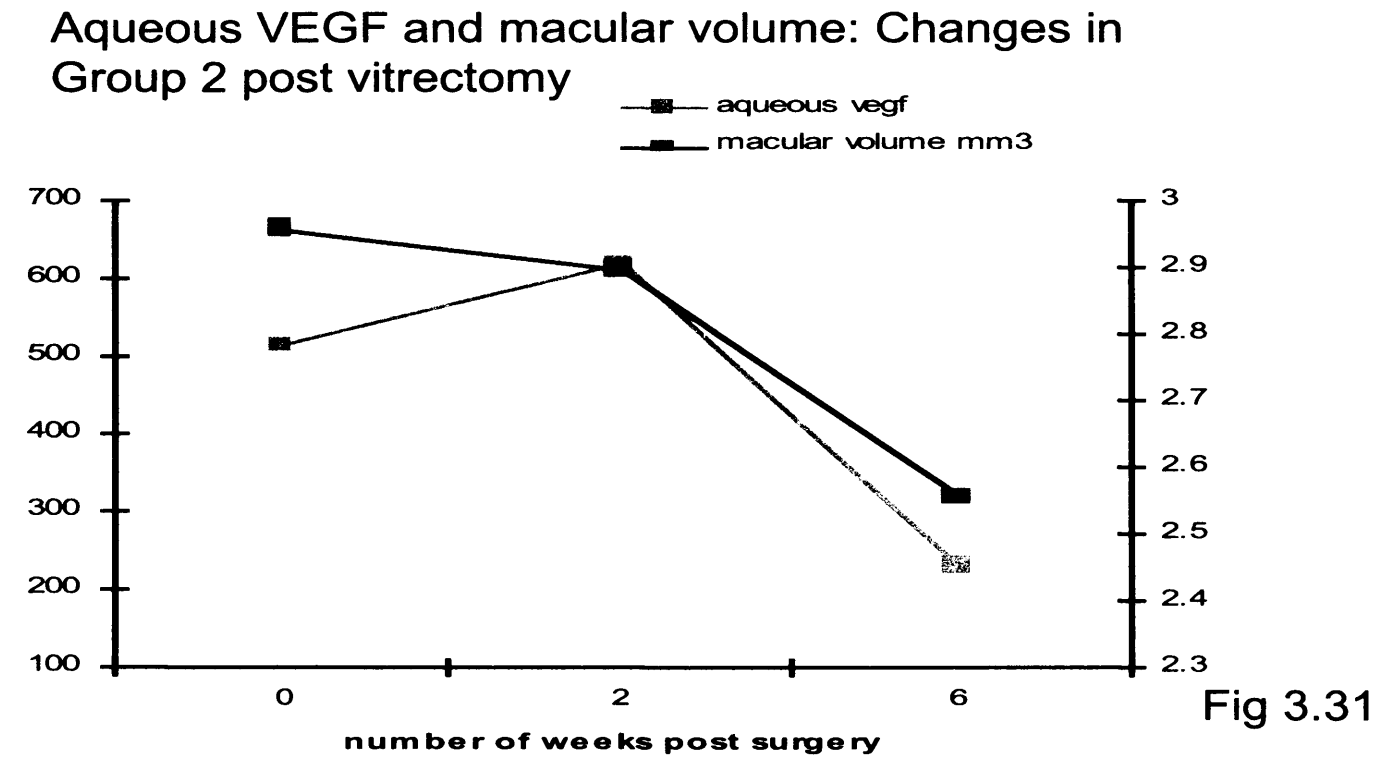


Fig 3.31

Figure 3.31: The changing aqueous VEGF concentrations and macular volumes post PPV in the two macular profile groups.

In Group 1 there is a decrease in macular volume as the aqueous VEGF concentration increases. In Group 2 both the macular volume and aqueous VEGF concentration decrease in tandem.

ANGIOPOIETIN LEVELS

The average duration of diabetes was 14 years (5-22 years) with HbA1C of 9% (9-12% in the diabetic patients whose average age was 58 (40-72 years). All had type II diabetes. Those patients with non-proliferative retinopathy also demonstrated clinically significant macular oedema that had been present for at least 15 months (12-18 months) prior to pars plana vitrectomy. They had received on average 3 macular laser treatments (range 1-6). Those with proliferative disease had received extensive pan-retinal photocoagulation and underwent PPV for delamination of membranes and further endolaser.

The standard curve of the LIA from which the sample concentrations were determined for both angiopoietin 1 and angiopoietin 2 **(Figure 3.32a and b respectively).**

The median vitreous angiopoietin 1 concentration for 5 patients with full thickness macular hole (FTMH) used as a control population was 150 pg/ml (range 3-227 pg/ml) compared to 1,933 pg/ml (range 289-5,820 pg/ml) in 17 patients with non-proliferative diabetic retinopathy (NPDR) and 186 pg/ml (range 26-2,292 pg/ml) for 10 patients with proliferative diabetic retinopathy (PDR) **(Figure 3.33 a) (Table 3.6).** The angiopoietin 1 concentrations in NPDR were higher than in FTMH ($p<0.001$) and PDR ($p<0.01$) whilst there was no difference in the angiopoietin 1 concentration between PDR and FTMH (ns).

The median angiopoietin 2 concentration in NPDR was 3,874 pg/ml (range 1,341-9,888 pg/ml) whilst in FTMH and PDR patients, angiopoietin 2 concentrations were below the level of sensitivity for the assay. The angiopoietin 1 concentration in NPDR was half that of angiopoietin 2 with the difference significant at $p<0.02$ **(Figure 3.33 b) (Table 3.6).**

Table 3.8 Vitreous concentrations of Angiopoietins (pg/ml) (<DL= below detection limit of assay; *= significance p< 0.05 compared to control).

	FTMH (control)	NPDR	PDR
Angiopoietin 1	150	1933 *	186
Angiopoietin 2	<DL	3874	<DL

Further, on comparing the foveal thickness to the angiopoietin 1 concentration in patients with NPDR, all of who had persistent CSMO, there was a non-significant trend towards a negative correlation ($p<0.08$ and $R= - 0.44$) (**Figure 3.34**). No such trend was seen on comparing angiopoietin 2 with these macular structural indices.

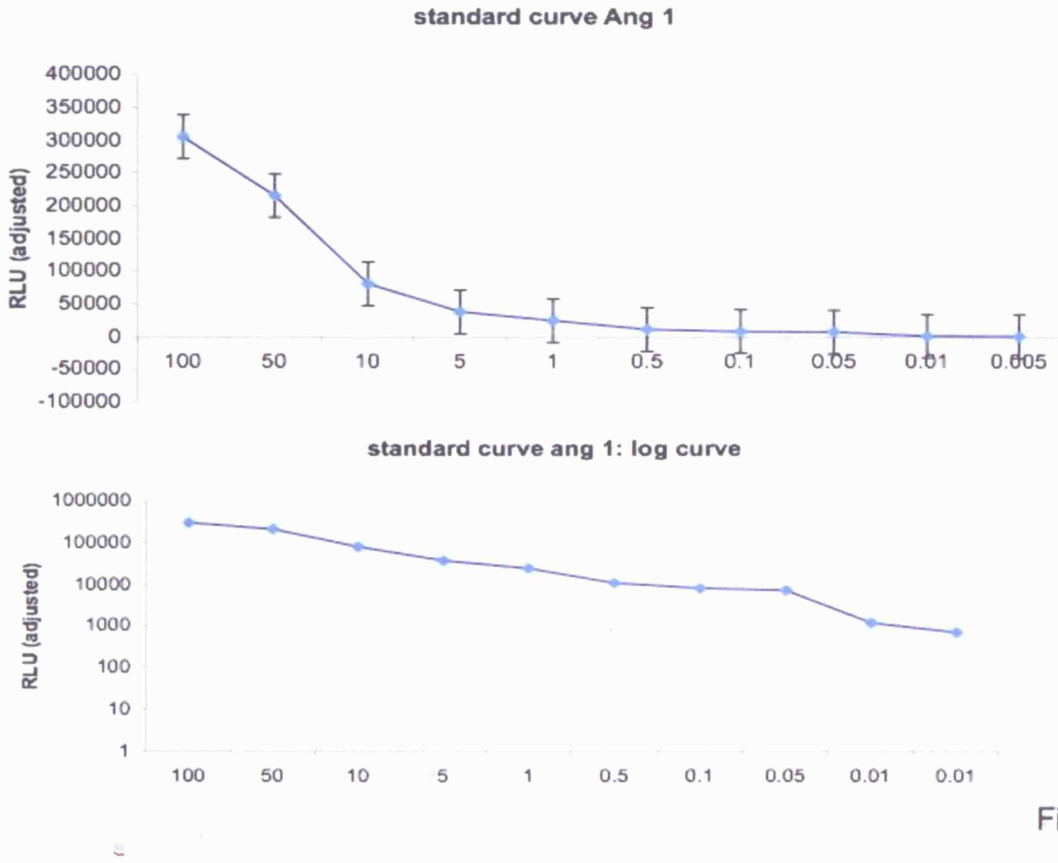


Fig 3.32a

Figure 3.32a: Standard Curve for Angiopoietin 1.

Sample concentrations were determined from this curve.

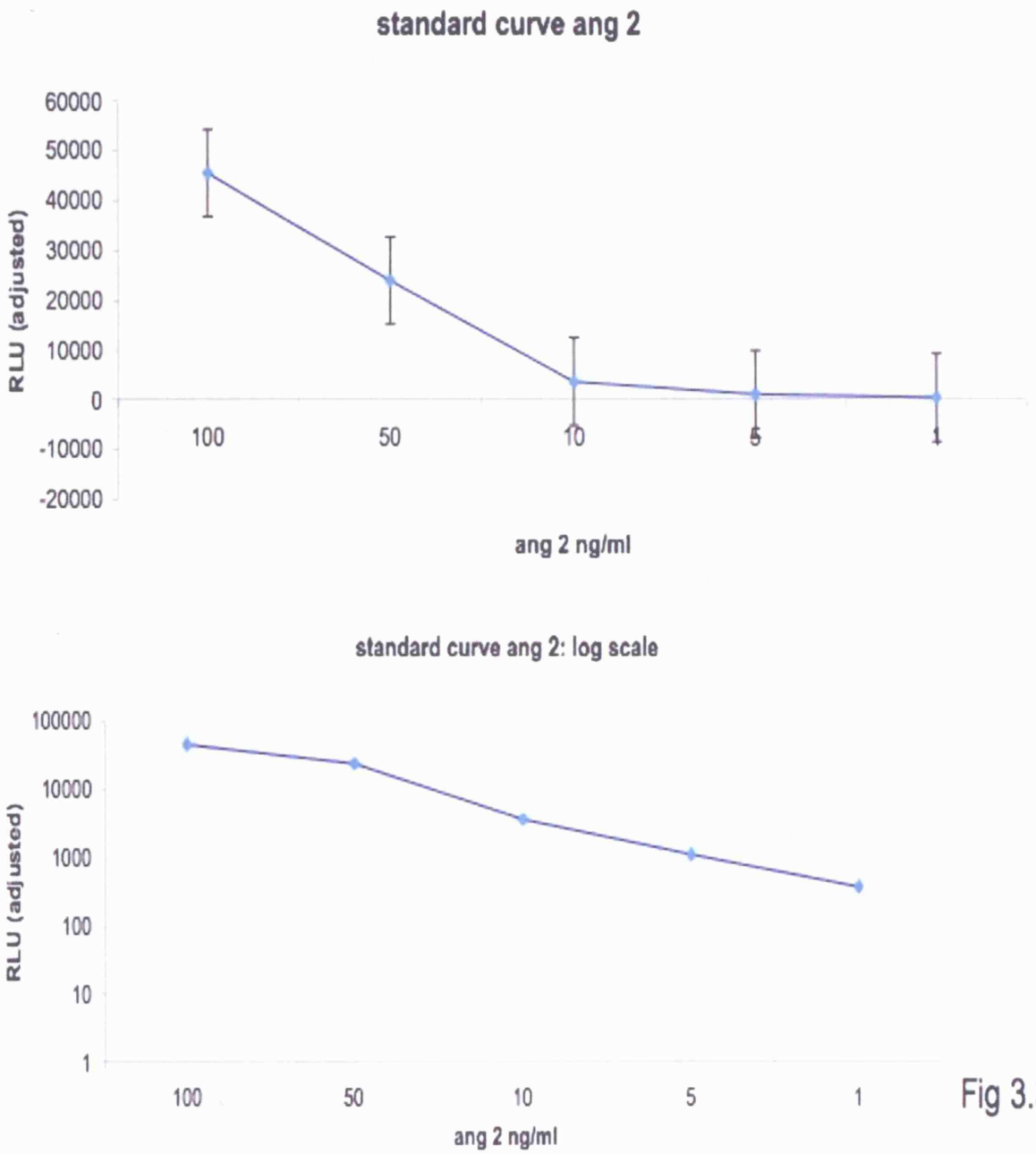


Figure 3.32b: Standard Curve for Angiopoietin 2.

Sample concentrations were determined from this curve.

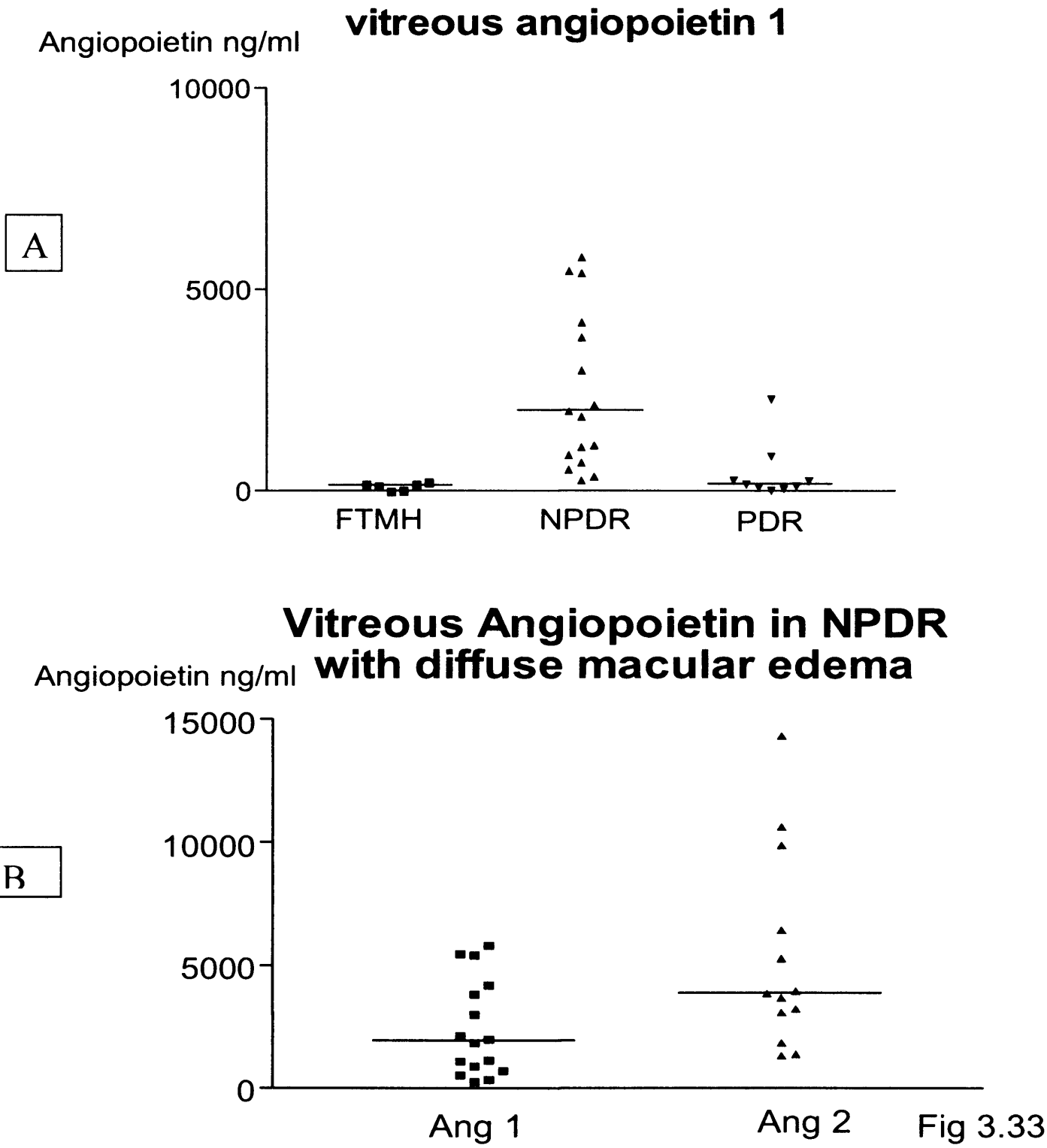


Figure 3.33: Vitreous angiopoietin concentrations in the diabetic state.
(FTMH= full thickness macular hole; NPDR= study patients with nonproliferative retinopathy and diffuse clinically significant macular oedema; PDR= proliferative diabetic retinopathy)

a) The angiopoietin 1 concentration in NPDR were higher than in FTMH ($p<0.001$) and PDR ($p<0.01$) whilst there was no difference in the angiopoietin 1 concentration between PDR and FTMH (ns). b) The angiopoietin 1 concentration in NPDR was half that of angiopoietin 2 with the difference significant at $p<0.02$

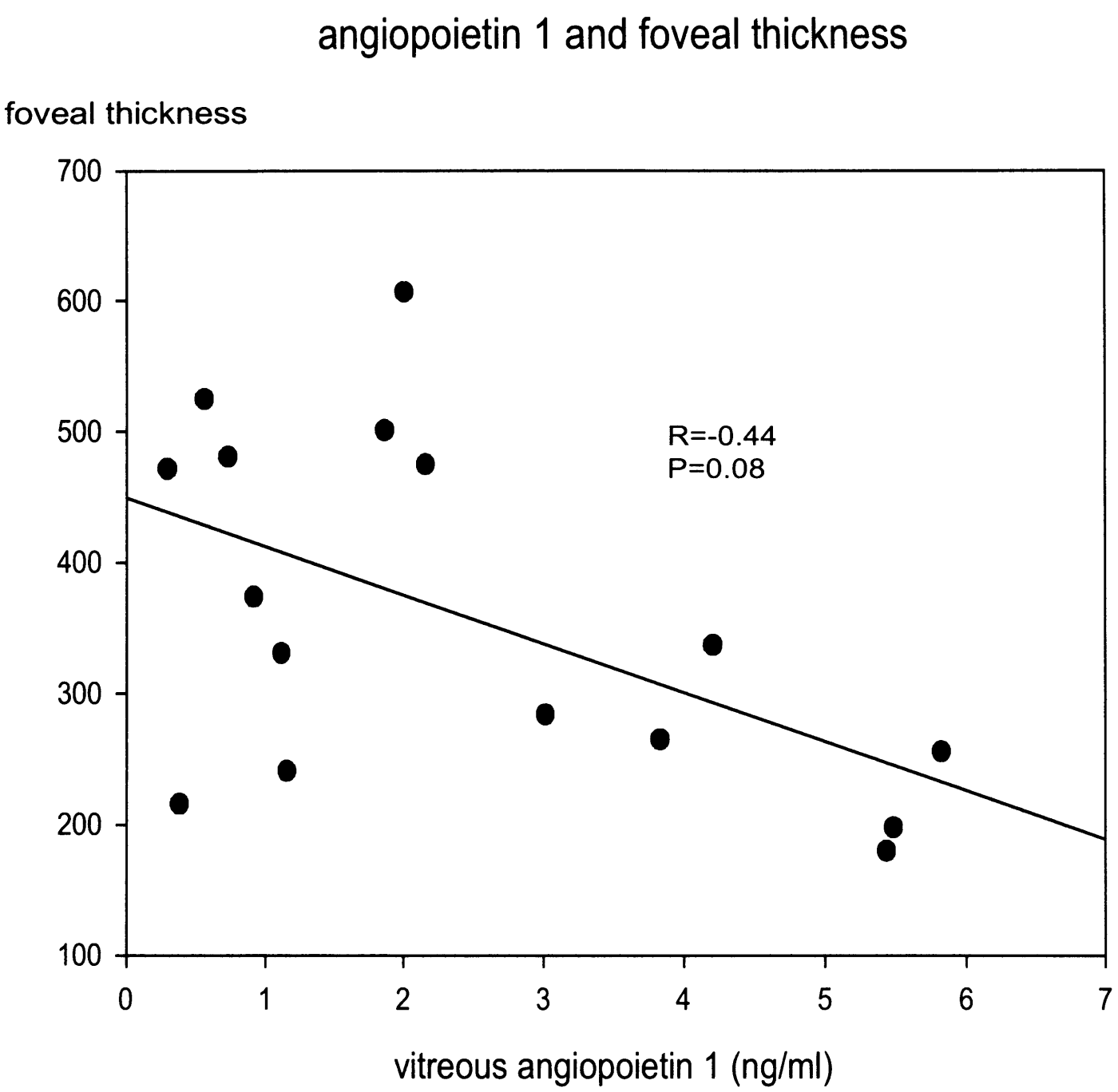


Fig 3.34

Figure 3.34: Negative correlation of vitreous angiopoietin 1 with foveal thickness.

R=-0.44, p=0.08 Spearman correlation test

In two patients there was sufficient post-operative aqueous available (after initially being used to determine VEGF concentrations) to evaluate post-operative the aqueous angiopoietin concentration over a 6 week period. This concentration varied according to whether there was a coincidental increase or decrease in the foveal thickness post surgery. In one patient there was a fall in the foveal thickness, with the angiopoietin 1 concentration increasing from a baseline of 845 pg/ml to 3,839 pg/ml at 6 weeks as the foveal thickness changed from 216 to 260 μm over the same period, but at 3 months the thickness had decreased to 174 μm (**Figure 3.35**). The angiopoietin 2 concentration remained relatively static (3,223 to 3,591 pg/ml over the period of observation). However, in the second patient, the foveal thickness increased and the angiopoietin 1 concentration decreased over the 6 week period (2,752 to 1,828 pg/ml) whilst angiopoietin 2 showed a reciprocal increase from 3,069 to 6,079 pg/ml over the same period (**see Figure 3.36**).

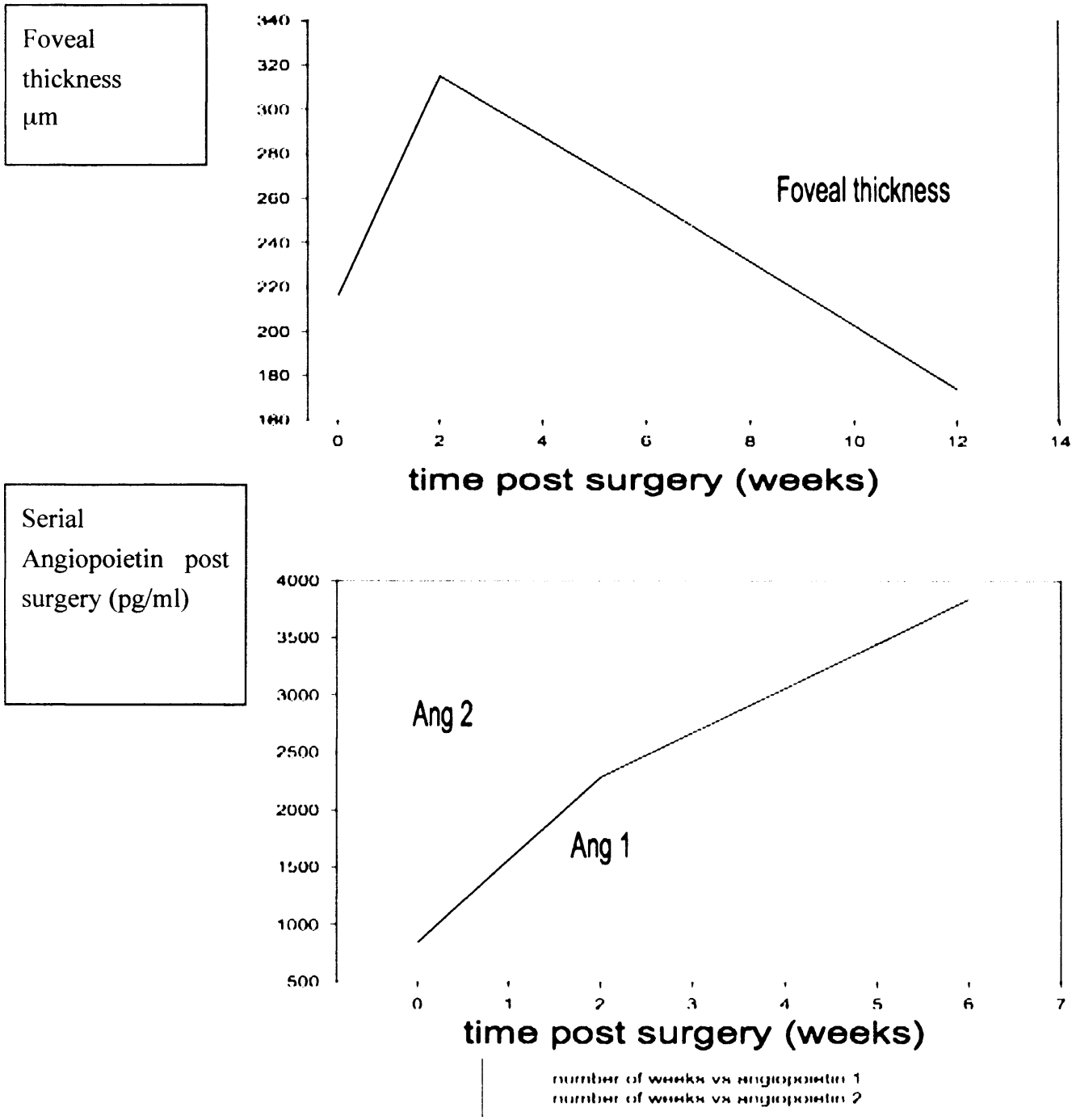


Fig 3.35

Figure 3.35: Improved foveal thickness as aqueous angiopoietin 1 increases post pars plana vitrectomy

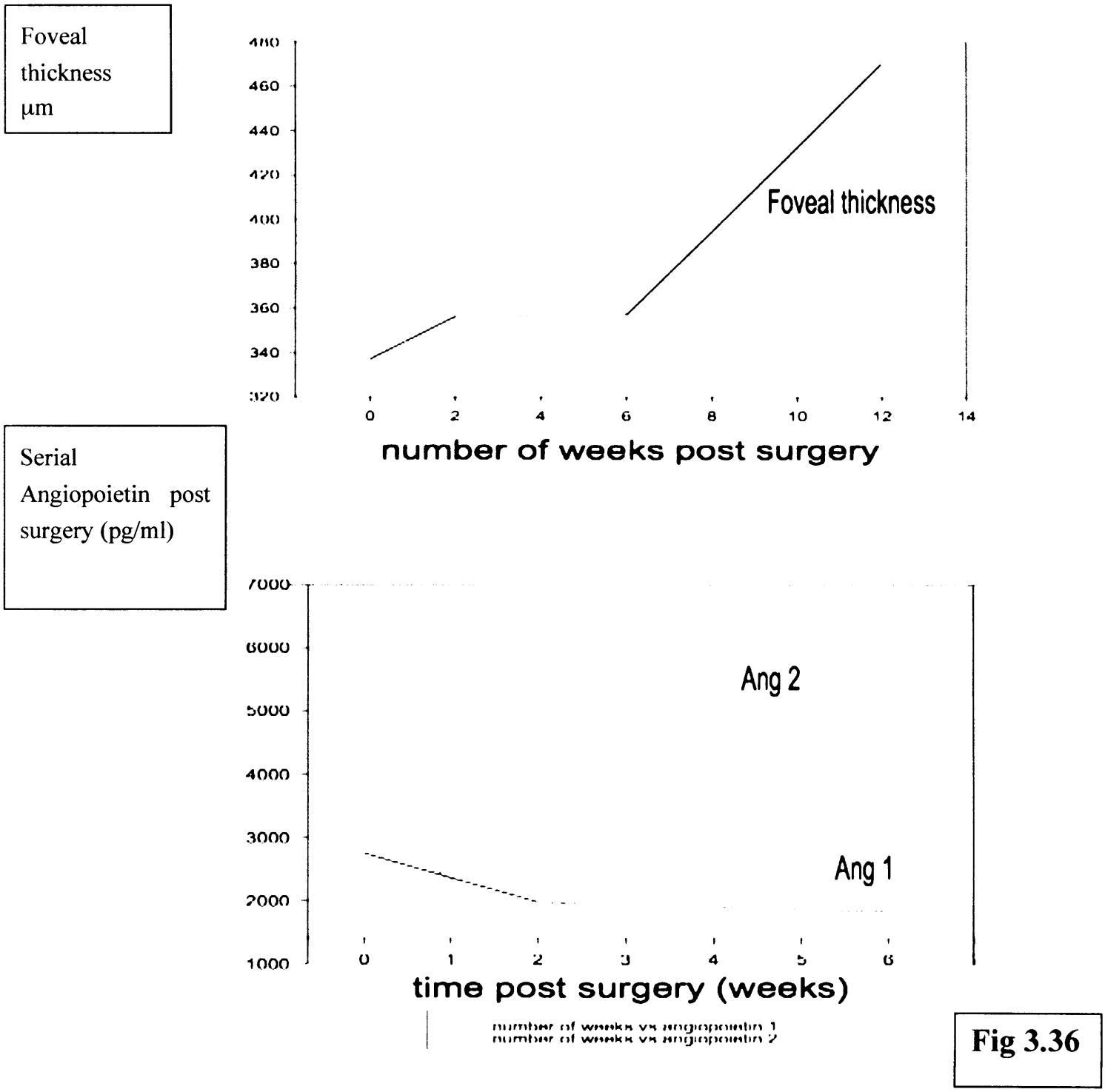


Figure 3.36: Worsening of foveal thickness as aqueous angiopoietin 2 increases post pars plana vitrectomy.

Chapter 3.2.2. HAEMODYNAMIC AND INFLAMMATORY CYTOKINES

Vitreous samples from five full thickness macular hole (FTMH) patients representing normal control were analyzed together with the test vitreous samples of 15 patients with non-proliferative diabetic retinopathy and five with proliferative diabetic retinopathy (PDR). The NPDR group was graded as moderate to severe non-proliferative, and all with persistent clinically significant macular oedema (CSMO) despite previous macular argon laser treatment.

In the NPDR group the average age of the fifteen patients was 64 years (range 46-72 years), all with type II diabetes mellitus and an average duration of 12 years (range 4-23 years). The average HbA1c was 8% (range 5.8-9.9), eight suffered from systemic hypertension, which was well controlled, and one was on lipid lowering drugs.

Where sufficient vitreous was available, a complete set of growth factor analysis for each patient enrolled was performed and in only such circumstances was the correlation analysis with clinical parameters carried out.

Vitreous Nitric Oxide (Total Nitrite)

The median concentration in FTMH patients was 85 μmol /l (range 67-87 μmol / l) whilst in the non-proliferative retinopathy (NPDR) with clinically significant macular oedema (CSMO) group the concentration was lower at 77 μmol / l (range 51-

118 μmol / l) and in proliferative retinopathy patients it was 73 μmol / l (range 40-100 μmol / l). There were no significant differences in the results between these groups (p=0.6) (**Figure 3.37**).

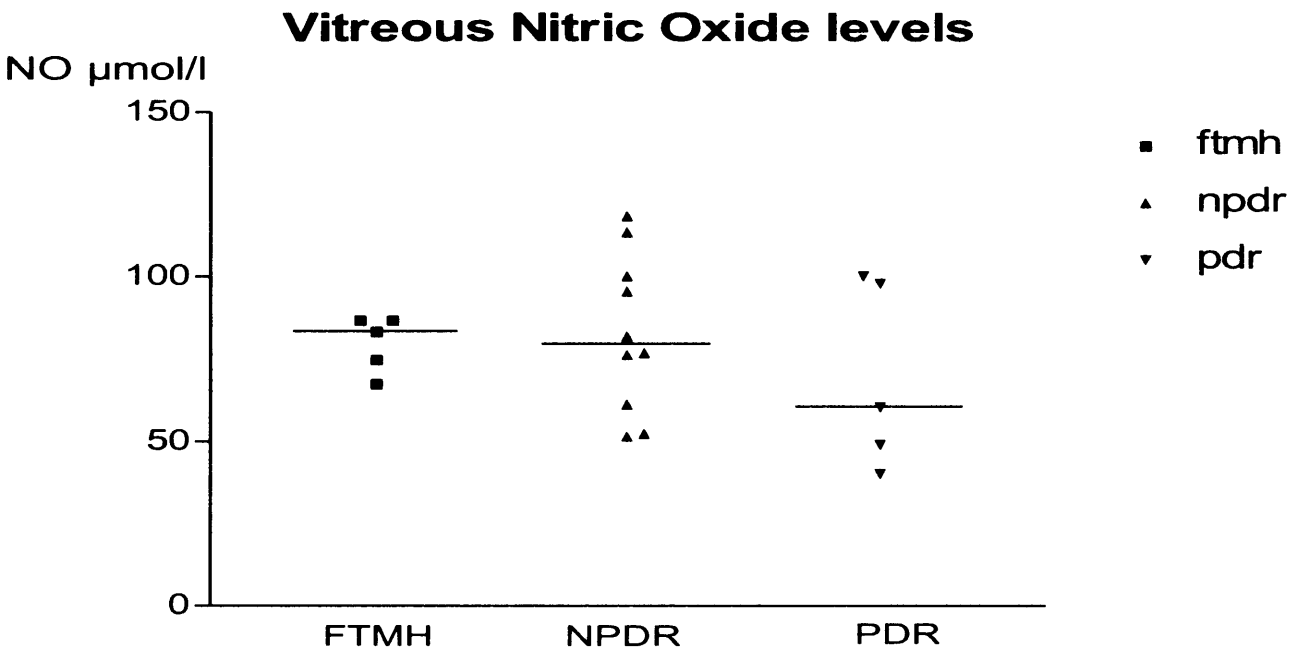


Fig 3.37

Figure 3.37: Vitreous Nitric Oxide levels in the Diabetic compared to control. There was no significant difference in the vitreous concentration between the groups. (ftmh= full thickness macular hole; npdr= nonproliferative diabetic retinopathy; pdr= proliferative diabetic retinopathy)

Vitreous Prostacyclin

The median level in macular hole patients was 49 pg/ml (range 48-52 pg/ml) whilst in the two diabetic patient groups the concentration of prostacyclin was similar to each other and also lower than the control patients (45 pg/ml in NPDR and in PDR (range 38-53 pg/ml). Even though there was a lowering of concentration in diabetes, this was not significant (p=0.0885) (**Figure 3.38**).

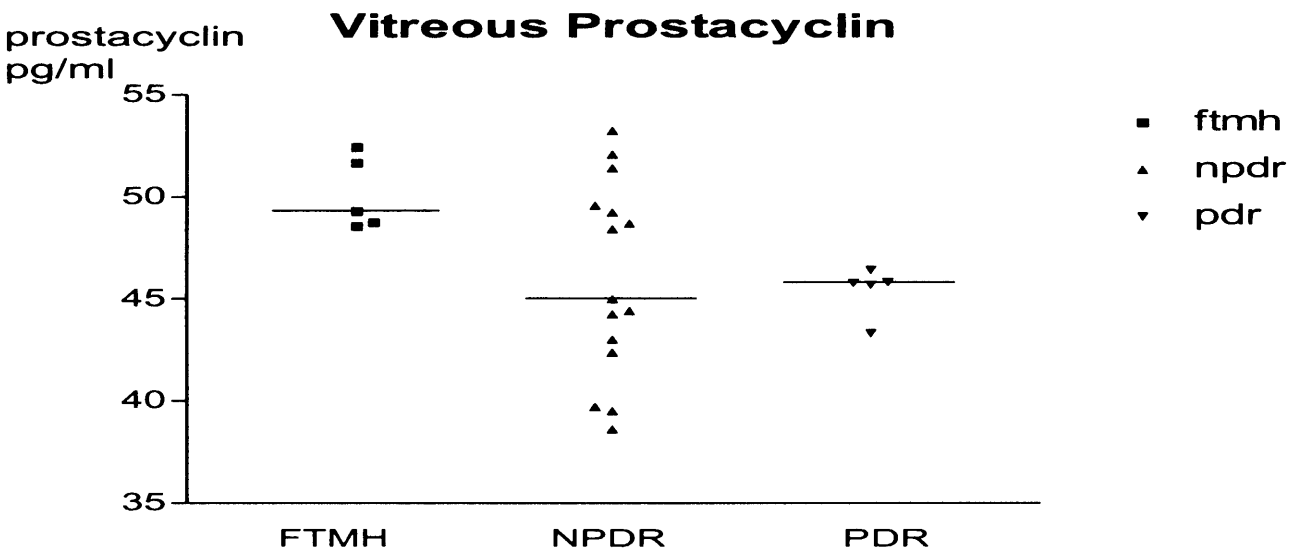


Fig 3.38

Figure 3.38: Vitreous Prostacyclin levels in the Diabetic compared to control.

There was no significant difference in the vitreous concentration between the groups. (ftmh= full thickness macular hole; npdr= nonproliferative diabetic retinopathy; pdr= proliferative diabetic retinopathy)

Vitreous Endothelin-1 levels

The median level in macular hole patients was 3.6 pg/ml (range 3.57-3.9 pg/ml) which decreased in NPDR with CSMO to 0.6 pg/ml (range 0.4-2.57 pg/ml) but increased in PDR to 6.25 pg/ml (range 5.5-7.24 pg/ml). The difference in the concentrations between the macular hole and NPDR ($p<0.0001$) and macular hole and PDR ($p<0.008$) was significant as was the difference between the two diabetic groups ($p<0.001$) (**Figure 3.39**).

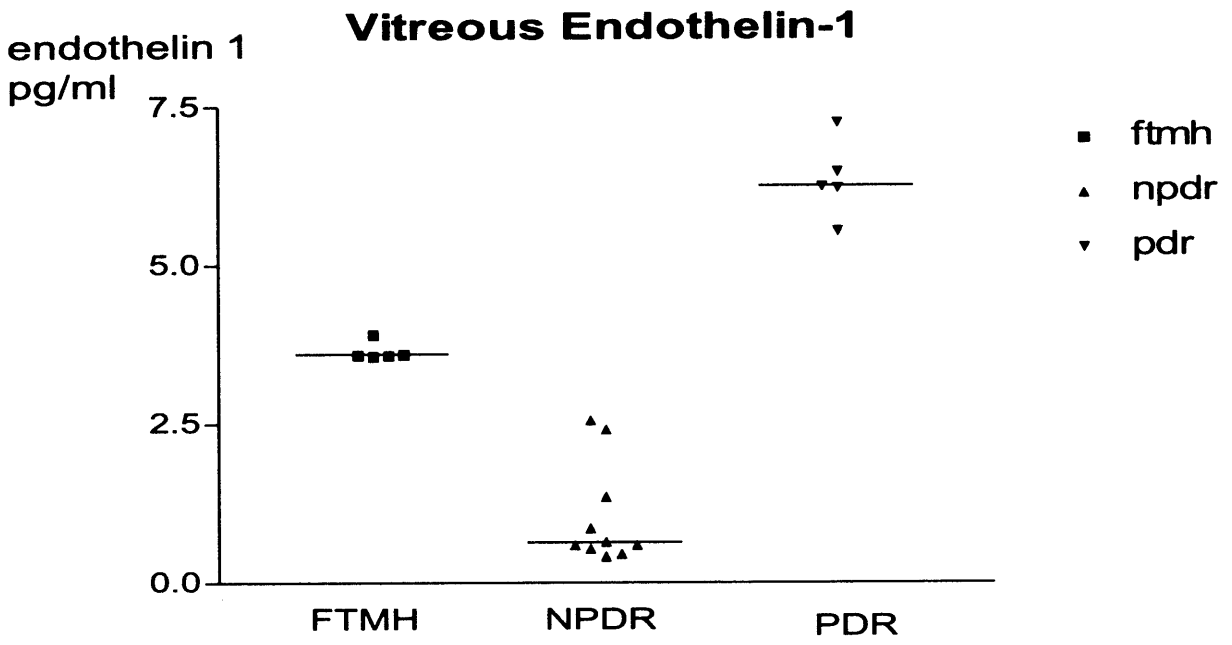


Fig 3.39

Figure 3.39: Vitreous Endothelin-1 levels in the Diabetic compared to control.

The difference in the concentrations between the macular hole and NPDR ($p<0.0001$) and macular hole and PDR ($p<0.008$) was significant as was the difference between the two diabetic groups ($p<0.001$) (ftmh= full thickness macular hole; npdr= nonproliferative diabetic retinopathy; pdr= proliferative diabetic retinopathy)

The median foveal thickness in ten patients with NPDR with CSMO was 334 μm (range 198-751μm) whilst the macular volume was 3.24 mm^3 (range 2.13-6.42 mm^3). In these ten patients there was sufficient vitreous for a full set of growth factor analysis to perform a subsequent regression analysis.

On performing multiple regression analysis using clinical parameters, only endothelin-1 concentrations correlated with the macular structural indices: with foveal thickness (R^2= 72 %, p=0.04) (**Figure 3.40**) and with macular volume (R^2= 75 %, p=0.03) (**Figure 3.41**), suggesting that the greater the foveal thickness or macular volume, the higher the vitreous endothelin-1 levels in the patients with NPDR and macular oedema.

The sensitivity of the assay was 0.32 pg/ml

Correlation of foveal thickness and vitreous endothelin-1 in Diffuse Macular Oedema

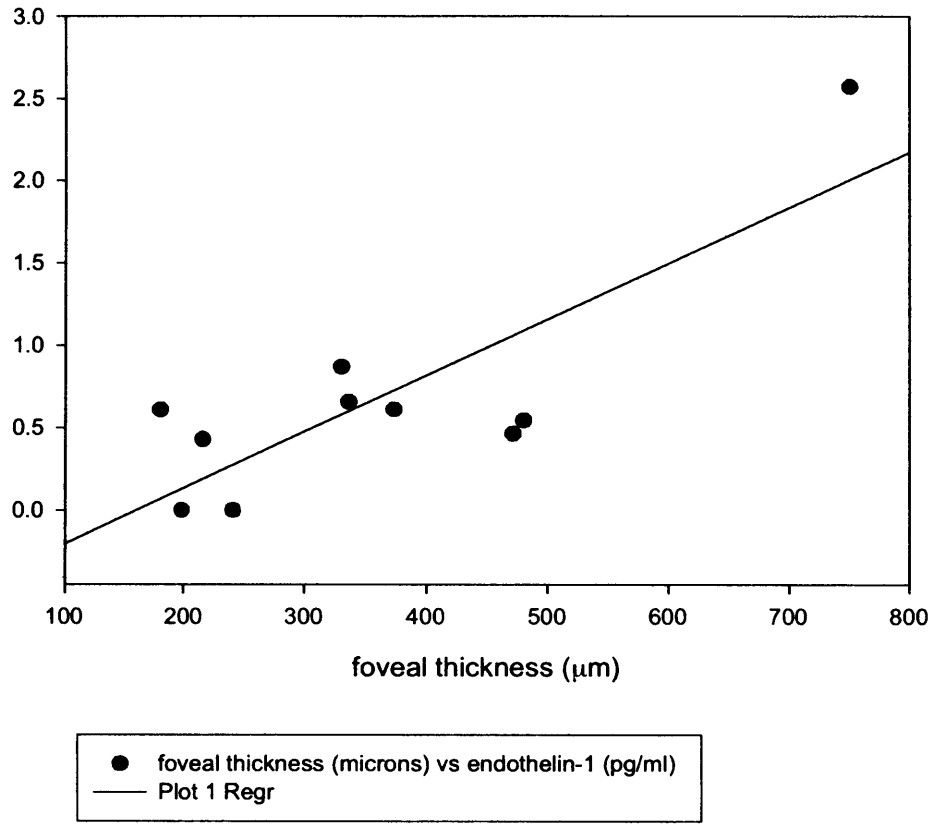


Fig 3.40

Figure 3.40: Correlation of Vitreous Endothelin-1 with foveal thickness in Diffuse Macular Oedema.

R^2= 72 %, p=0.04

Correlation of vitreous endothelin-1 with macular volume in Diffuse Macular Oedema

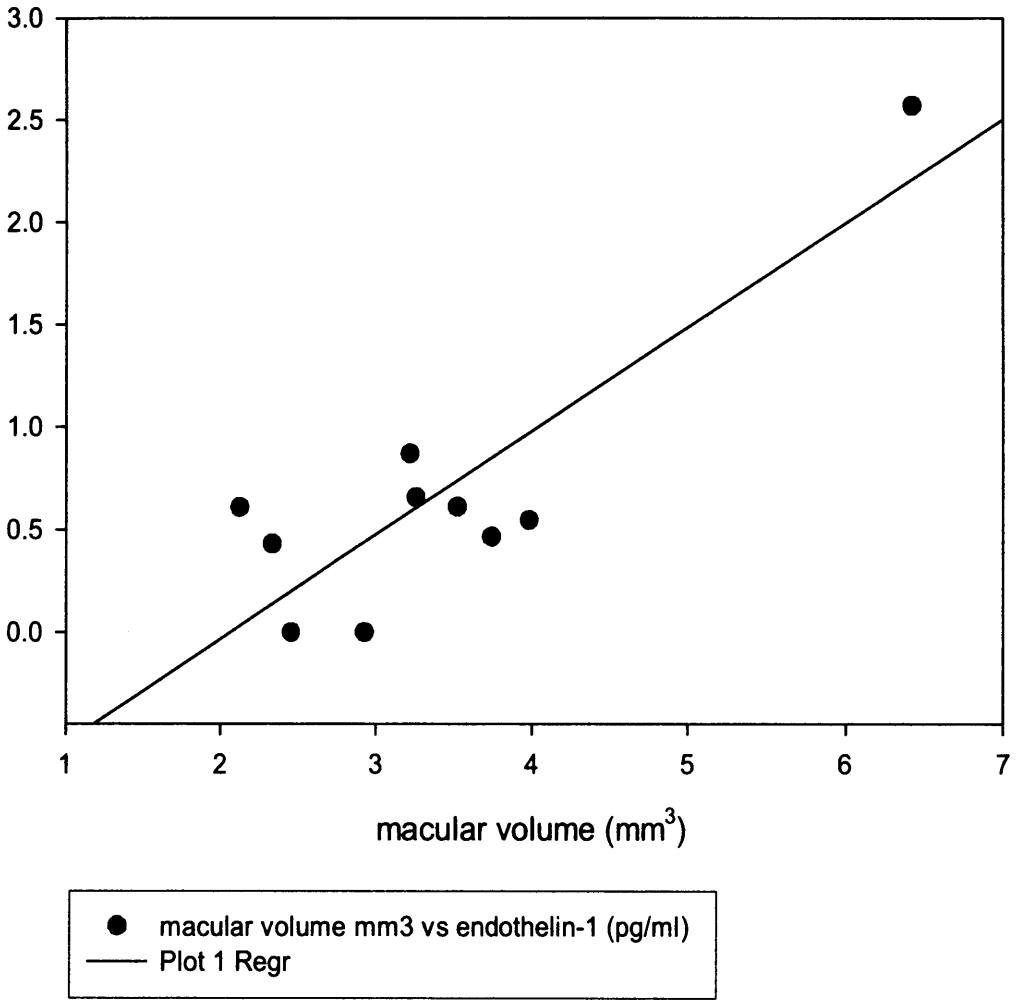


Fig 3.41

Figure 3.41: Correlation of Vitreous Endothelin-1 with macular volume in Diffuse Macular Oedema.

R^2= 75 %, p=0.03

Vitreous IL-1 beta levels

The standard curve of the LIA from which the sample concentrations were determined for both IL-1 β and IL-1 Ra (**Figure 3.42a and b respectively).**

The levels in the macular hole patients (representing normal controls) and NPDR with macular oedema patients were below the level of sensitivity of the assay (1 pg/ml) but the level in PDR was 5.58 pg/ml (2.1-6.3 pg/ml).

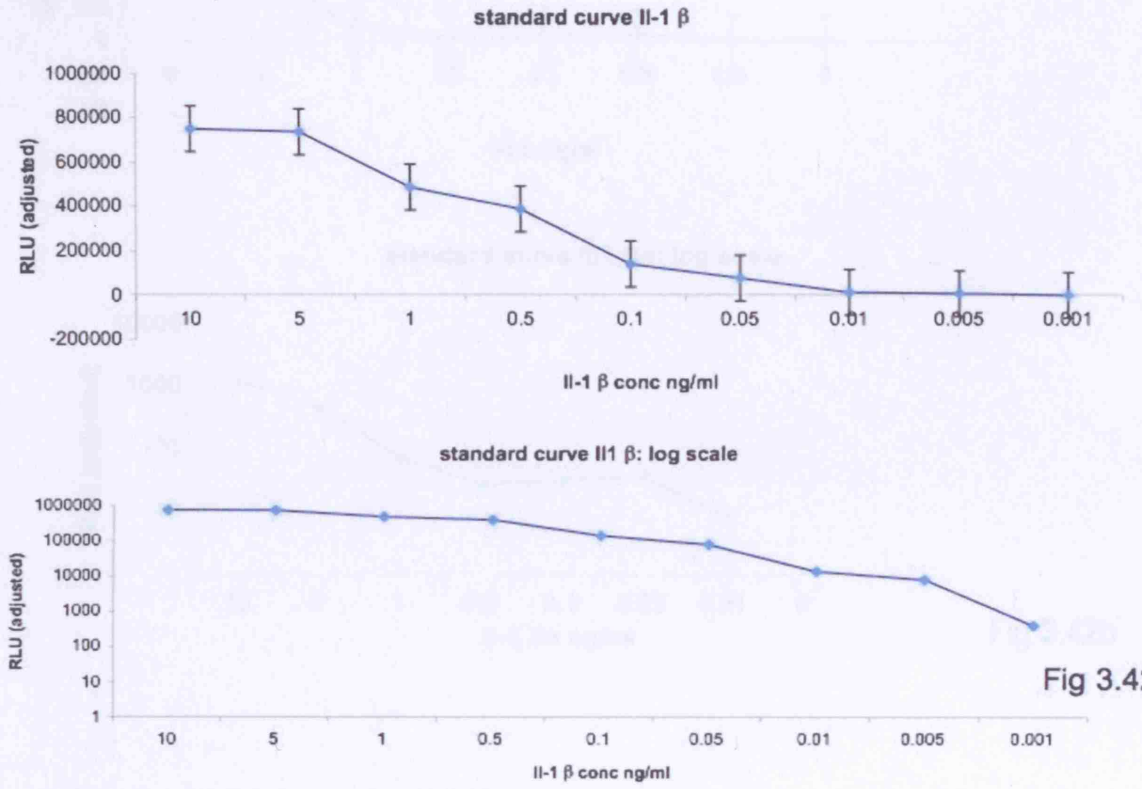


Fig 3.42a

Figure 3.42a: Standard Curve for Il-1 β.

Sample concentrations were determined from this curve.

Vitreous IL-1 Ra

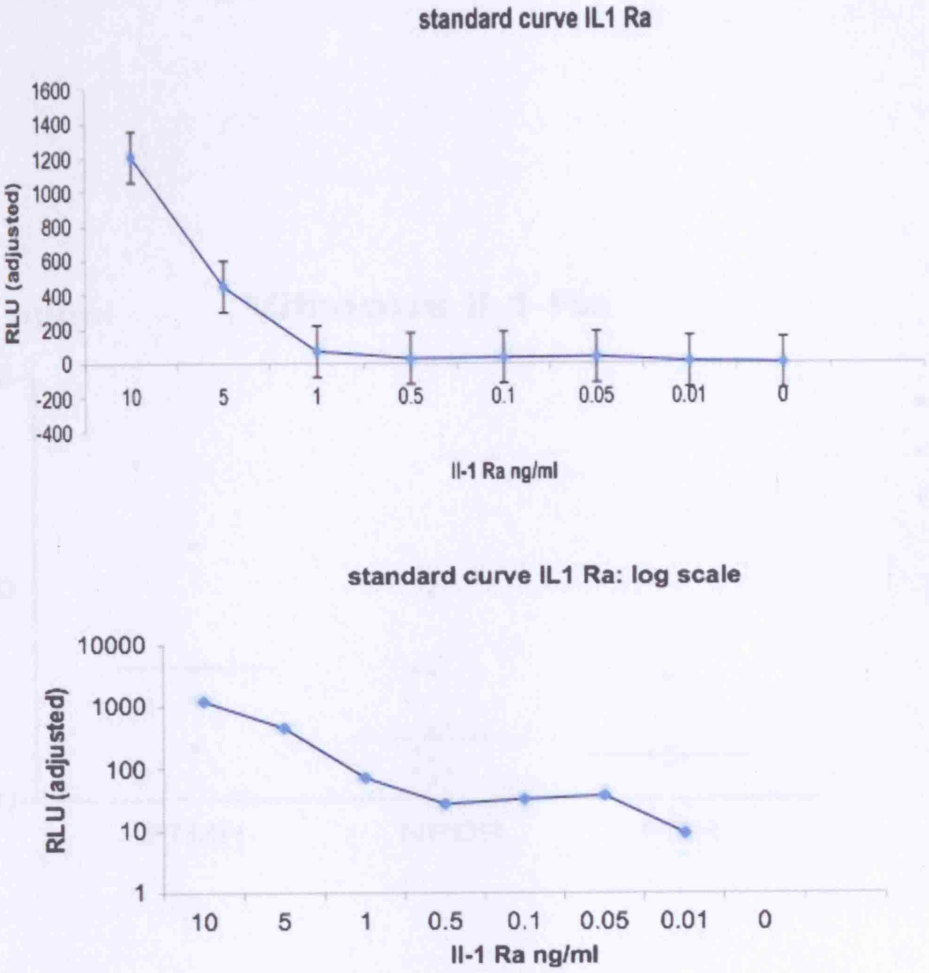


Fig 3.42b

Figure 3.42a: Standard Curve for Il 1 Ra.

Sample concentrations were determined from this curve.

The median concentration in macular hole patients was 6.23 ng/ml (range 2-12 ng/ml) and in NPDR and PDR the level was 2 ng/ml (range 0.9 to 9.72 ng/ml) (the sensitivity of the assay was 0.5 ng/ml). Given the wide variation in levels in the individual groups, the differences between the groups approached significance but failed to achieve it (p=0.057) (**Figure 3.43**).

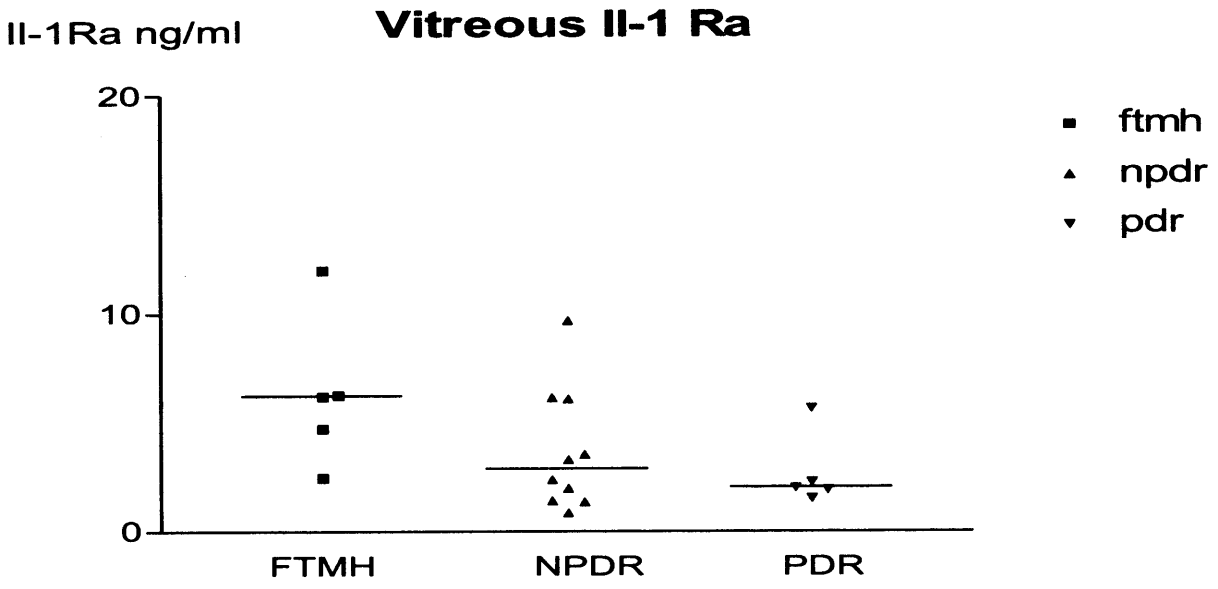


Fig 3.43

Figure 3.43: Vitreous Il-1 Ra levels in the Diabetic compared to control.

Given the wide variation in the concentrations in the individual groups, the differences between the groups approached significance but failed to achieve it (p=0.057)

Chapter 3.3 CELL CULTURE AND PERMEABILITY RESULTS

To investigate the possibility of using an immortalized rat endothelial cell line (JG2) to study the effects of cytokines to alter the spatial distribution of tight junctions, adherens junctions and caveolae, a study was undertaken to assess the effect of VEGF-A and HGF on the expression of these key cellular components in this cell line and compare it to primary rat endothelial cell cultures.

Chapter 3.3.1. IMMUNOCYTOCHEMISTRY

Chapter 3.3.1.1 JG 2 CELLS

ZONULA OCCLUDENS (ZO-1) STAINING

a) 30 MINUTE TREATMENT

When recombinant VEGF-A isoform 165 was applied for 30 minutes to JG 2 cultured cells, a concentration dependent effect was seen (**Figure 3.44**). At the lowest concentration (0.7 ng/ml) there was minimal disruption of ZO-1 junctional staining. At progressively greater concentrations (1.5ng/ml up to 100ng/ml) there was increasing loss and disruption of ZO-1 junctional staining. The two lowest concentrations used (0.7 ng/ml and 1.5 ng/ml) which represented concentrations of VEGF-A similar to those found in the vitreous of the patients recruited in the study (as described above), with 0.7ng/ml similar to the group of patients with a dome-shaped macular profile of oedema on OCT (Group 1) and 1.5 ng/ml similar to the diffuse-low elevation macular profile of oedema on OCT (Group 2). At 0.7 ng/ml VEGF-A, only minimal changes were seen in ZO-1 with no obvious difference to control in the distribution of ZO-1.

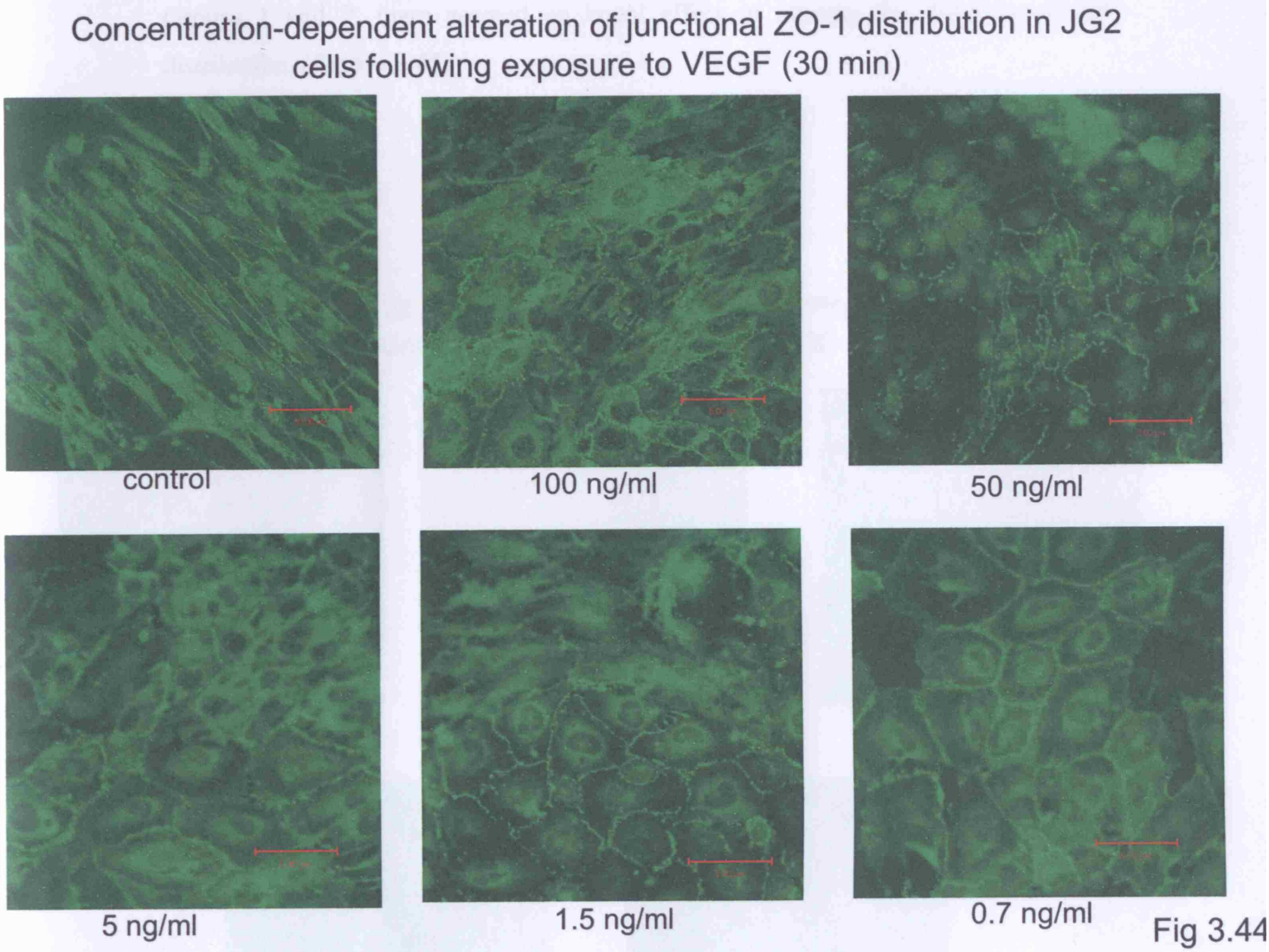


Fig 3.44. VEGF-A induces fragmentation and disruption of JG2 ZO-1 junctional protein, which is concentration dependent. Slide bar = 5μm

HGF at 20 ng/ml and 1.5 ng/ml demonstrated loss of ZO-1 junctional staining **(Figure 3.45)** as did the application of the vitreous from a patient in Group 1 and Group 2. It seemed that both HGF concentrations produced decreases in junctional staining to a

similar level but with the addition of vitreous from a representative sample from both Groups 1 and 2, there seemed an equal effect in altering the ZO-1 junctional distribution. **(Figure 3.45)**

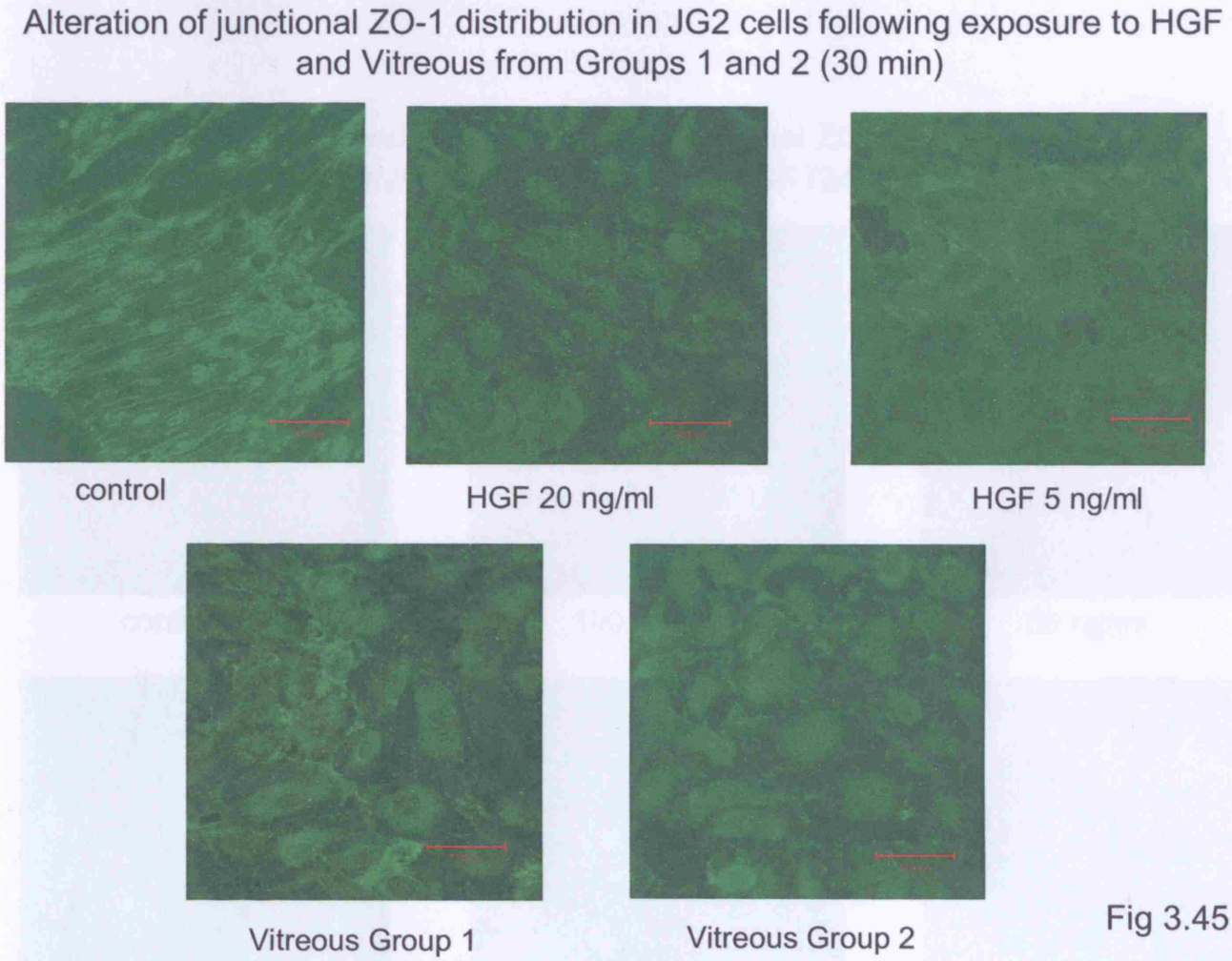


Fig 3.45. HGF and vitreous from Groups 1 and 2 also induce alterations in ZO-1 staining. Slide bar = 50 µm

b) 24 HOUR TREATMENT

Compared to untreated JG2 cells, 24 hour treatment with VEGF-A produced a relatively smaller decrease in the junctional staining of ZO-1 but a concentration dependent effect was still seen as before **(Figure 3.46)**. A similar effect was seen when HGF and a representative sample of vitreous from Groups 1 and 2 were used: there was disruption of ZO-1 staining **(Figure 3.47)**.

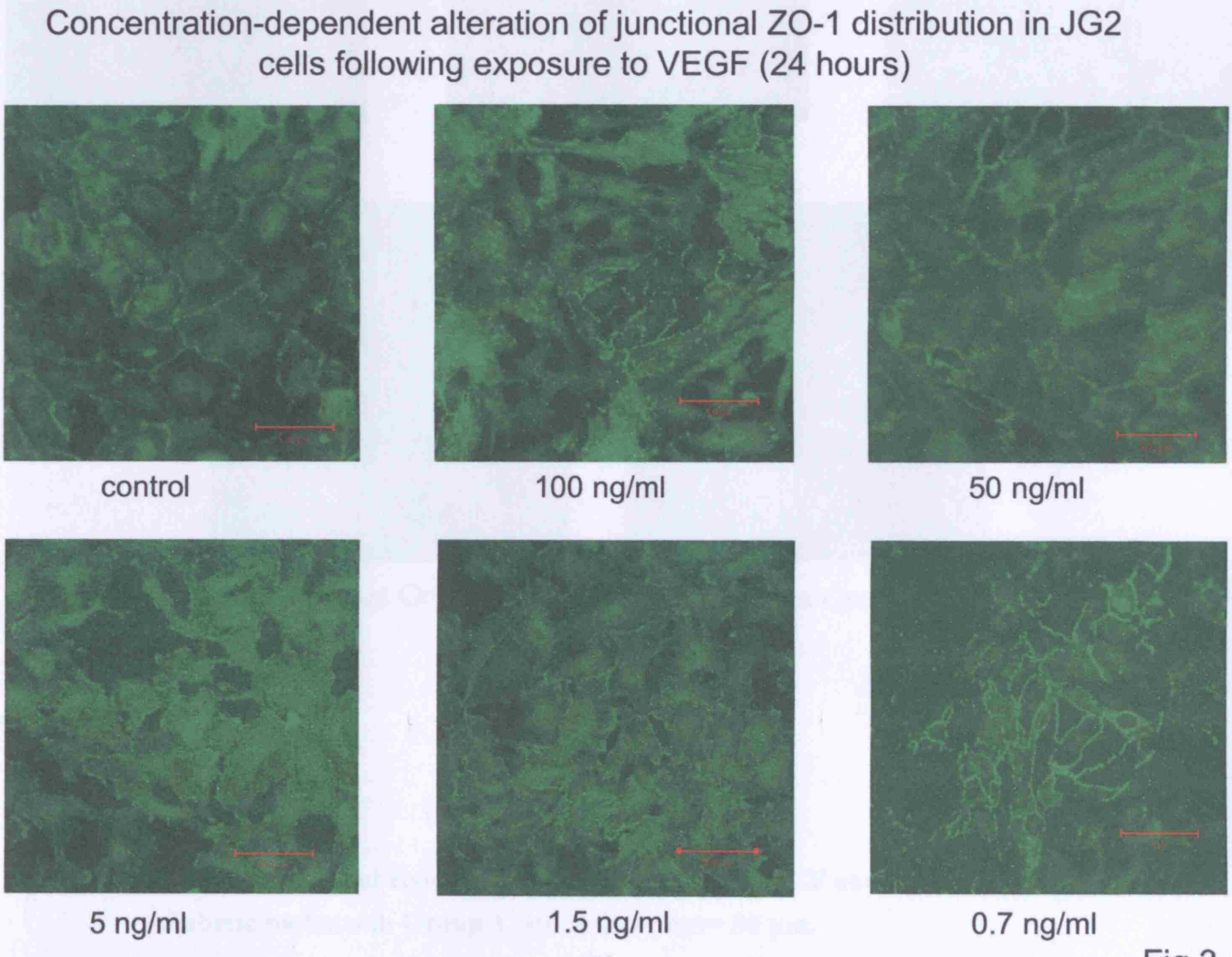


Fig 3.46. 24 hours of VEGF-A also induces alterations and decreased junctional staining of ZO-1, which is concentration dependent. Slide bar 5 µm.

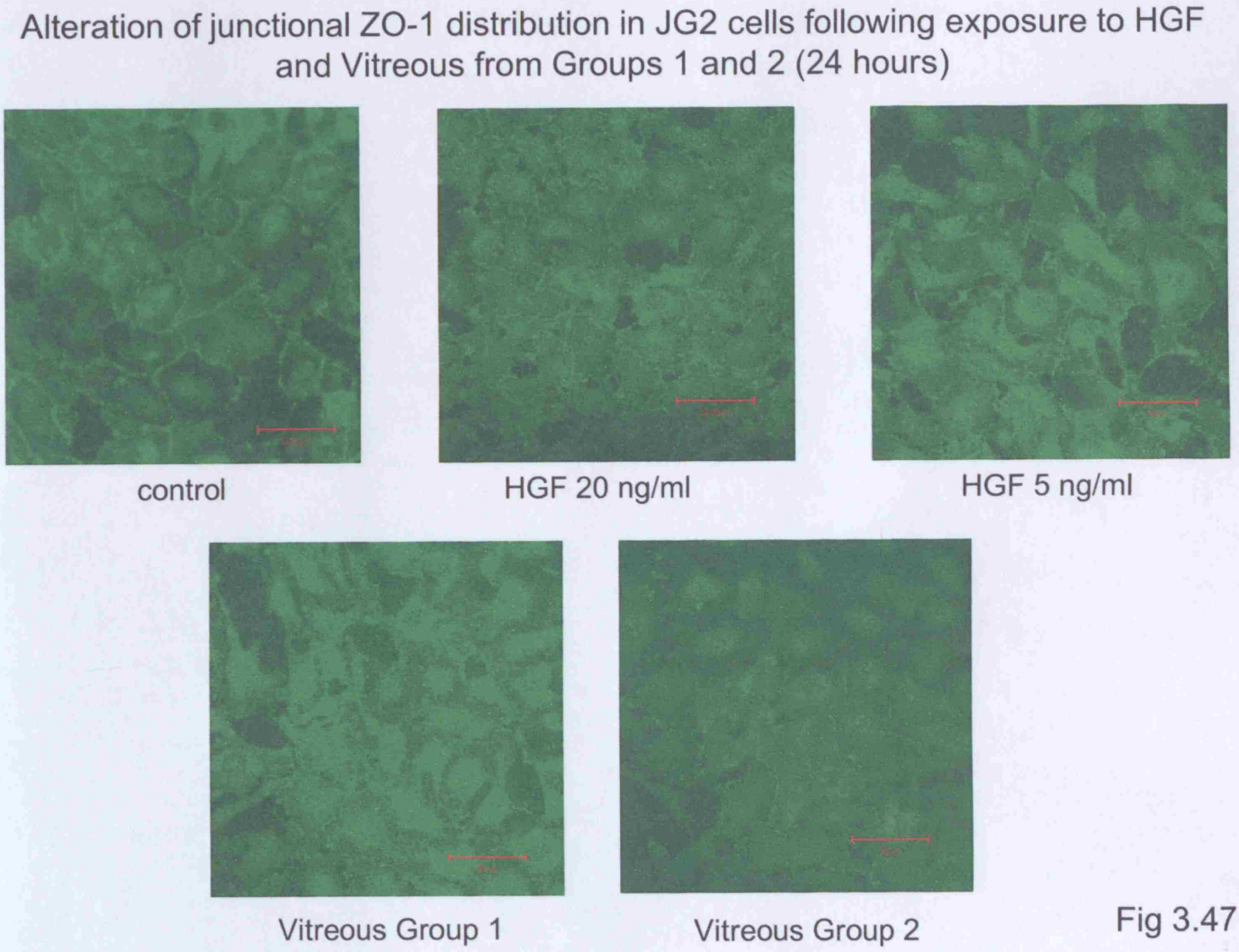


Fig 3.47. Changes and reduction of ZO-1 induced by HGF and vitreous from diabetic patients in Group 1 and 2. Slide bar= 50 µm.

Secondary only antibody staining acting on cells acting as negative control showed no junctional staining of ZO-1 (**Figure 3.48**)

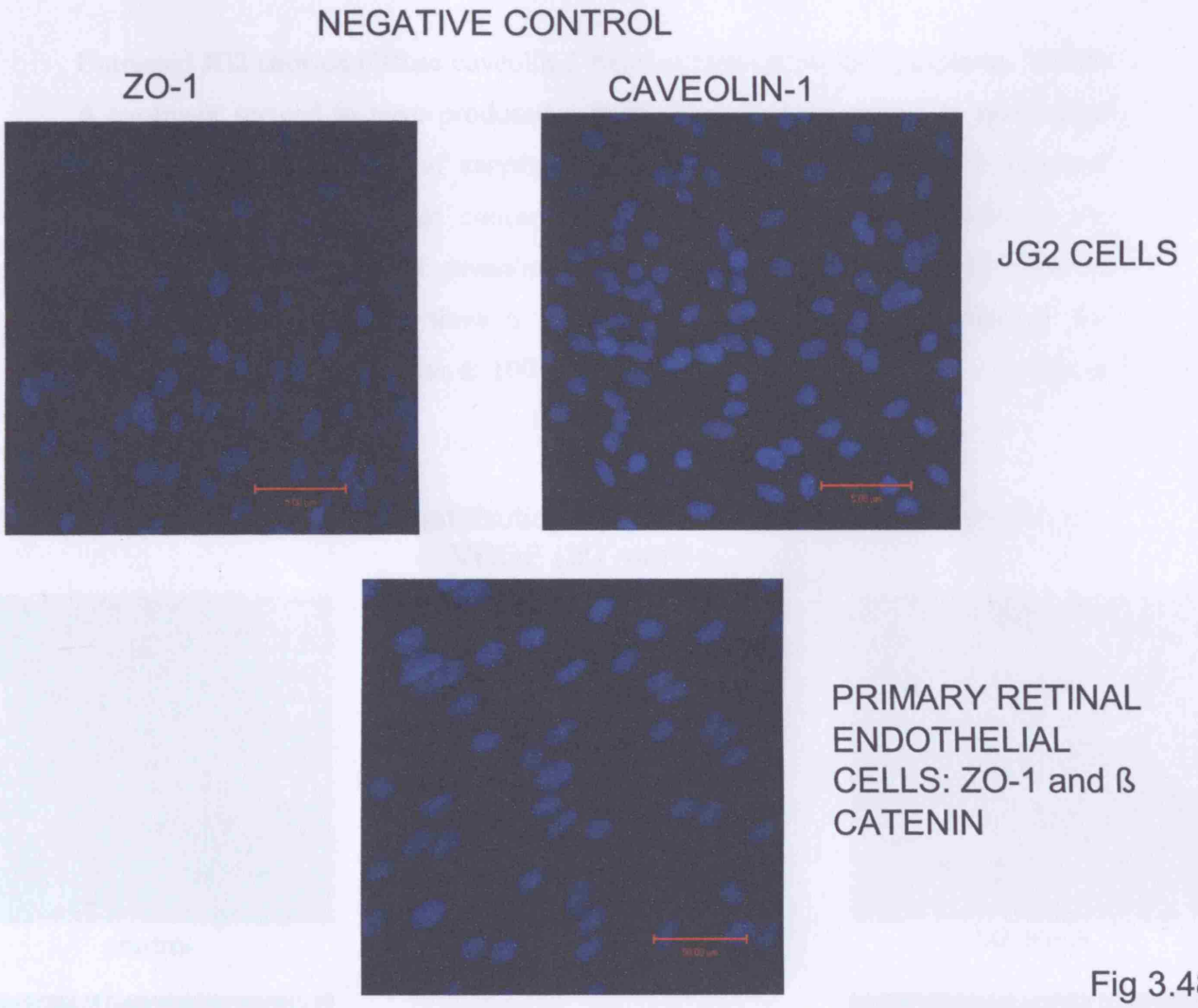


Fig 3.48: Negative control for JG2 and primary retinal endothelial cells. Slide bar = 5 µm.

The interpretation of the effects of VEGF on ZO-1 distribution needs to be qualified by the observation that the morphology of the JG2 cells were variable not only between different cultures but also within the same culture plate. This variation may reflect the quality of the cells and also the different ages of the cells used when plated out from the orginal frozen and stored sample.

CAVEOLIN-1 STAINING

c) 30 MINUTE TREATMENT

Untreated JG2 showed diffuse caveolin-1 staining throughout the cytoplasm. VEGF-A treatment seemed to have produced a redistribution of caveolin-1 to perinuclear staining ('perinuclear cap') of varying intensity and density compared to untreated cells. VEGF-A at the higher concentrations produced the greatest effect on the cytoplasmic redistribution of caveolin-1 compared to lower concentrations **(Figure 3.49)**. VEGF-A seemed to show a 'biphasic' response in this redistribution: the greatest effect seemed to occur at 100 ng/ml and 1.5 and 0.7 ng/ml, with no effect at 50 and 5 ng/ml VEGF-A.

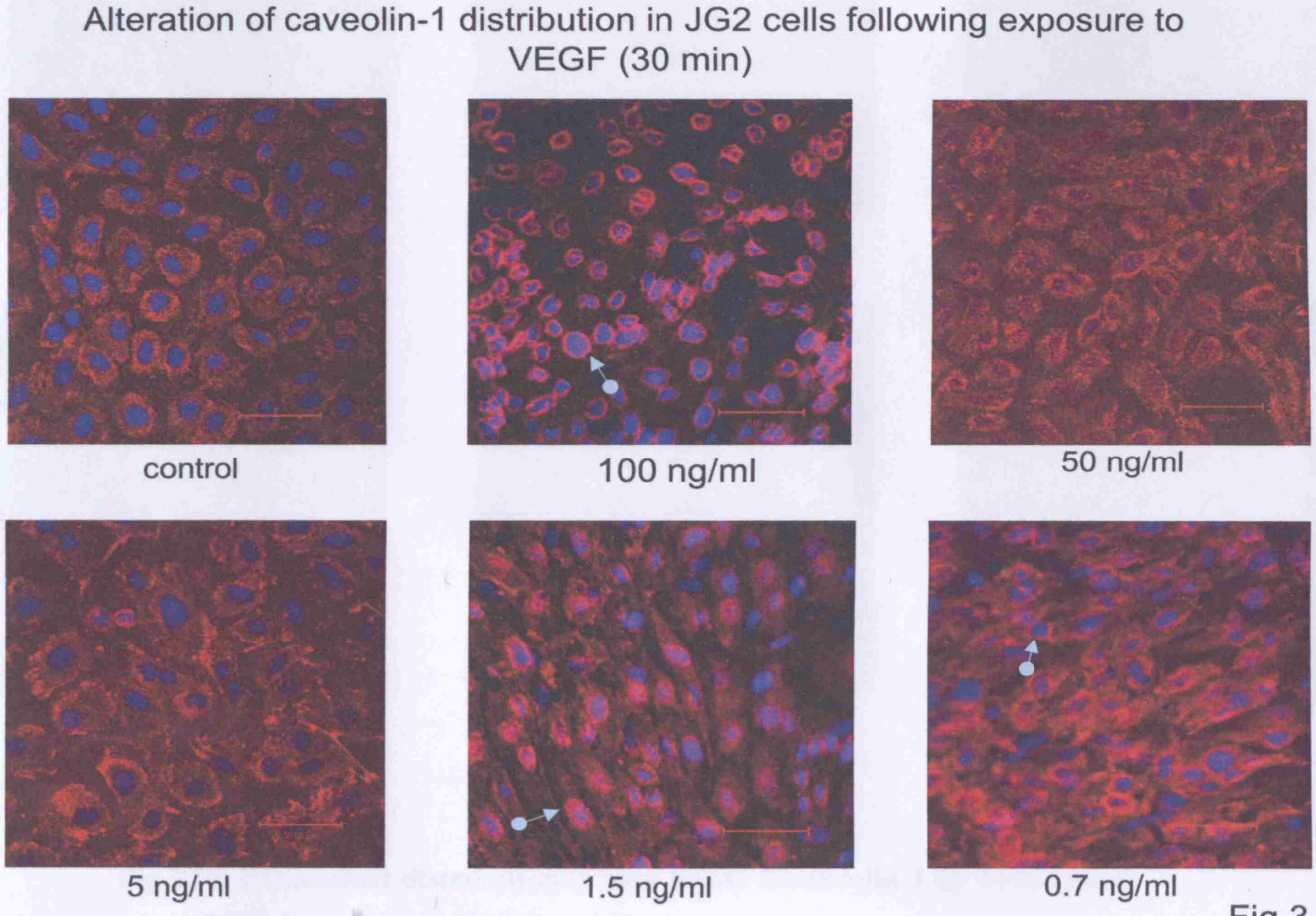


Fig 3.49

Fig 3.49. VEGF-A induces cytoplasmic redistribution of caveolin-1 to the perinuclear area forming the 'perinuclear cap' (arrow head). This seems to have a biphasic response. Slide bar = 5 μm

d) 24 HOUR TREATMENT

VEGF-A also demonstrated a biphasic response when the JG2 cells were exposed to VEGF-A for 24 hours: the perinuclear cap was seen at 100 ng/ml and the two lowest concentrations (**Figure 3.50**).

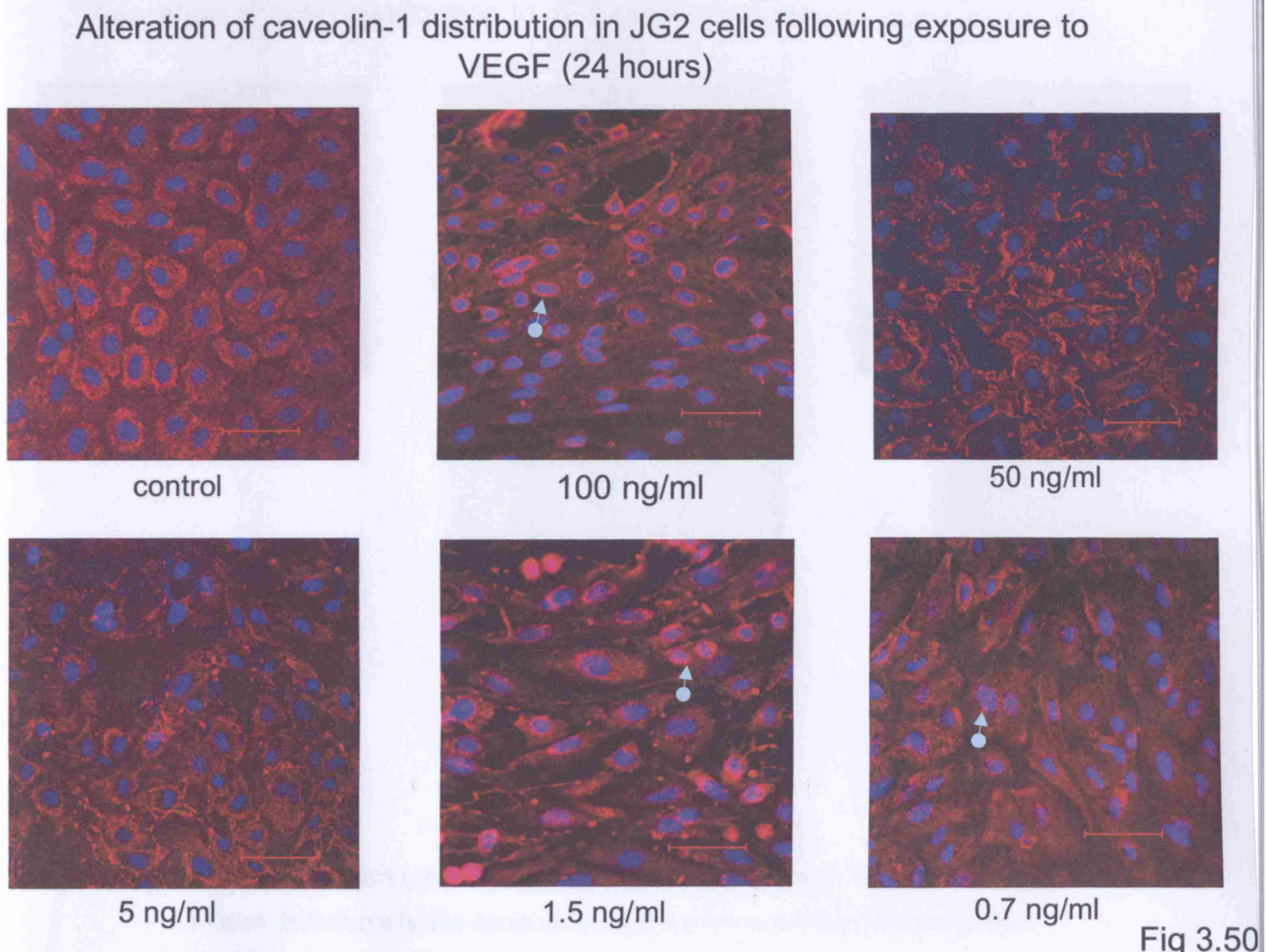


Fig 3.50. Perinuclear distribution (arrow head) of caveolin-1 by 24 hours of VEGF-A treatment. Slide bar = 5 μm

Secondary only antibody staining acting on cells acting as negative control showed no staining for caveolin-1 within the cells (**Figure 3.48**)

ACTIN STAINING

In both non treated and treated cells, staining of actin fibrils particularly around the cell border looked similar irrespective of which VEGF-A concentration was used. This similarity of staining was seen for both 30 minute and 24-hour time treatments of VEGF-A (**Figure 3.51 and 3.52**)

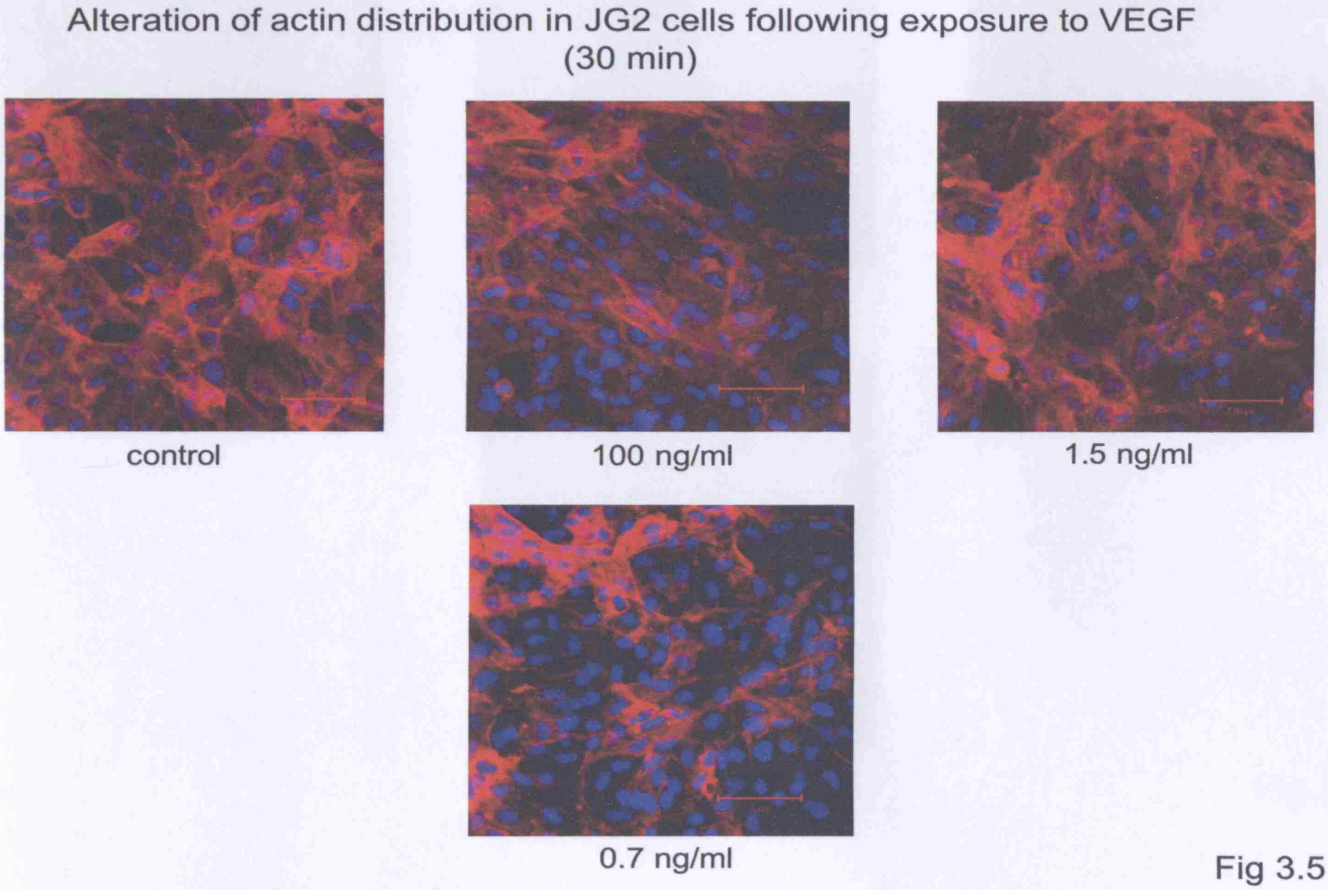


Fig 3.51

Fig 3.51. The effect of VEGF-A on actin distribution on JG2. There seemed no obvious difference in the actin staining between control and treated JG2 cells. Slide bar = 5 μm

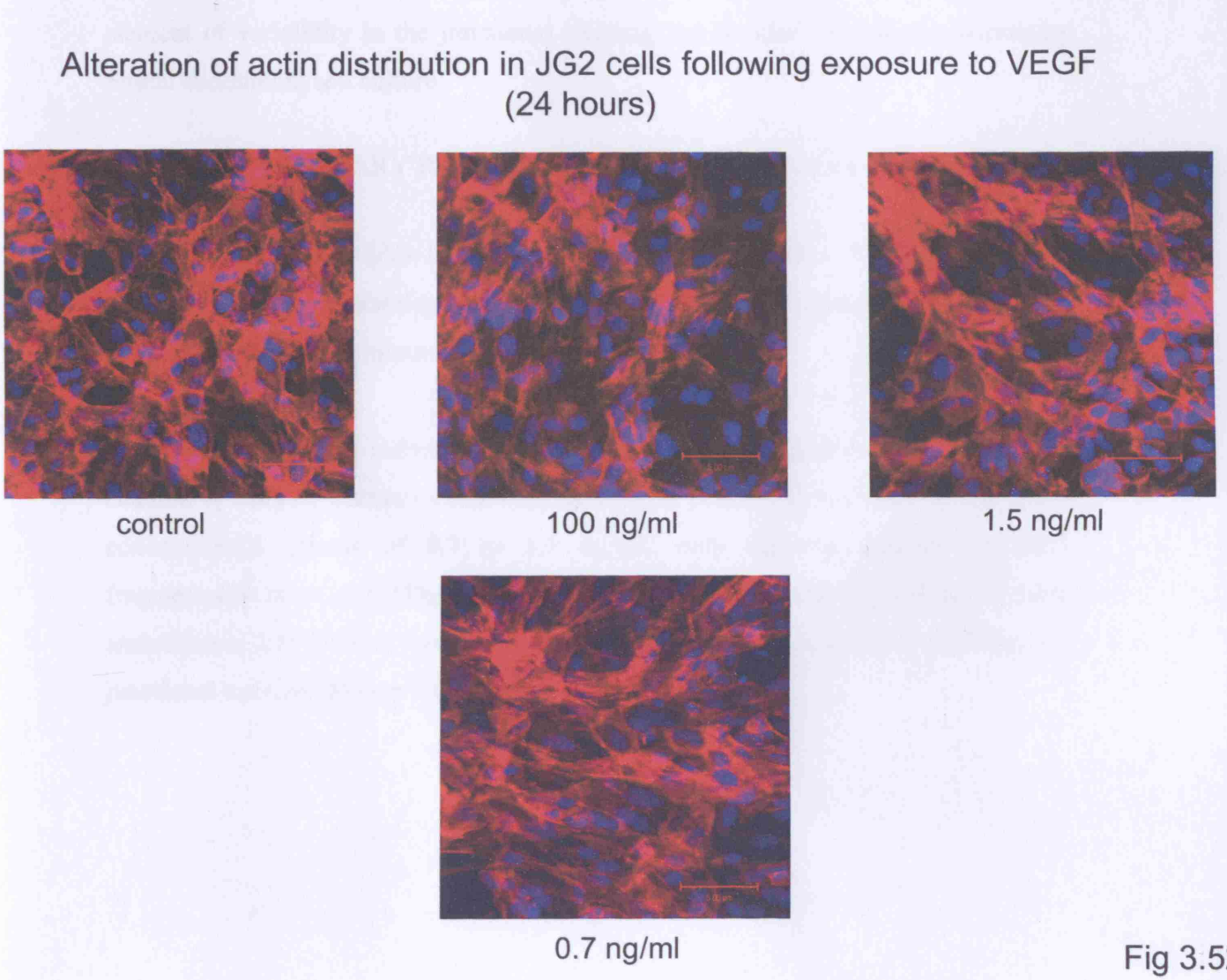


Fig 3.52. Actin staining. Similar to 30-minute treatment, no obvious difference in actin staining between control (untreated) and treated cells even after 24 hour treatment of VEGF-A. Slide bar = 5 μm

Secondary only antibody staining acting on cells acting as negative control showed no staining of actin fibrils **(Figure 3.48).**

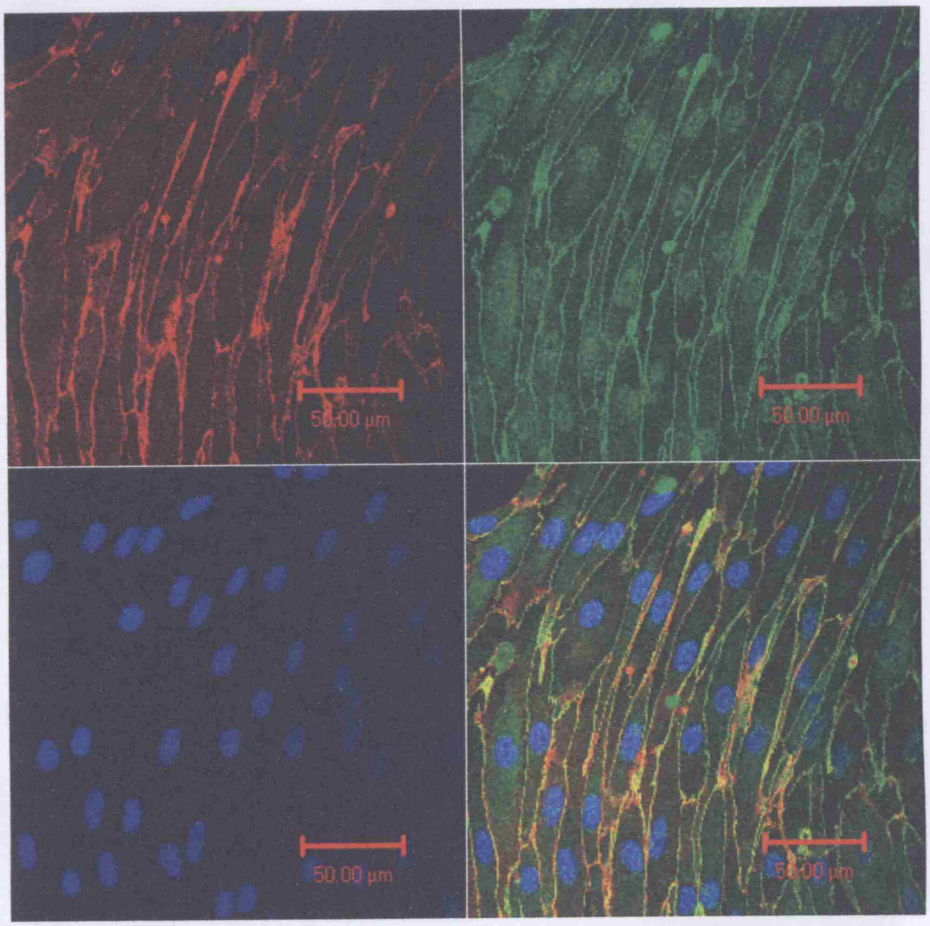
We used JG2 to determine if these cells could be used as a model to study the effects of VEGF-A and other cytokines on the junctional proteins. However given the element of variability in the junctional staining, we decided to progress to primary retinal endothelial cell culture.

Chapter 3.3.1.2 PRIMARY RAT RETINAL ENDOTHELIAL CELLS

Immunocytochemistry (ZO-1 staining is seen in green and red represents β-catenin immunocytochemical staining) with normal staining seen in **Figure 3.53.** All cytokine treatments were for 30 minutes. The scale bar is 50 μm.

VEGF-A at 100 ng/ml induced, reorganization of the junctional protein ZO-1 and ß-catenin. It became discontinuous with less linear junctional staining. At the lower concentrations (levels of 0.7 to 1.5 ng/ml) only minimal changes of ZO-1 fragmentation were seen **(Figures 3.54 to 3.56)**. Similarly with the addition of HGF and vitreous from Groups 1 and 2 there was minimal disruption of ZO-1 and ß-catenin junctional staining **(Figure 3.57-3.60)**.

CONTROL PRIMARY RAT RETINAL ENDOTHELIAL CELLS



Red= ß catenin
Green= ZO-1
Blue= nucleus
Overlay

Fig 3.53

Fig 3.53. Normal distribution of ß catenin and ZO-1 with overlay image for primary rat retinal endothelial cells.

The effect of VEGF 100 ng/ml on primary retinal endothelial cell

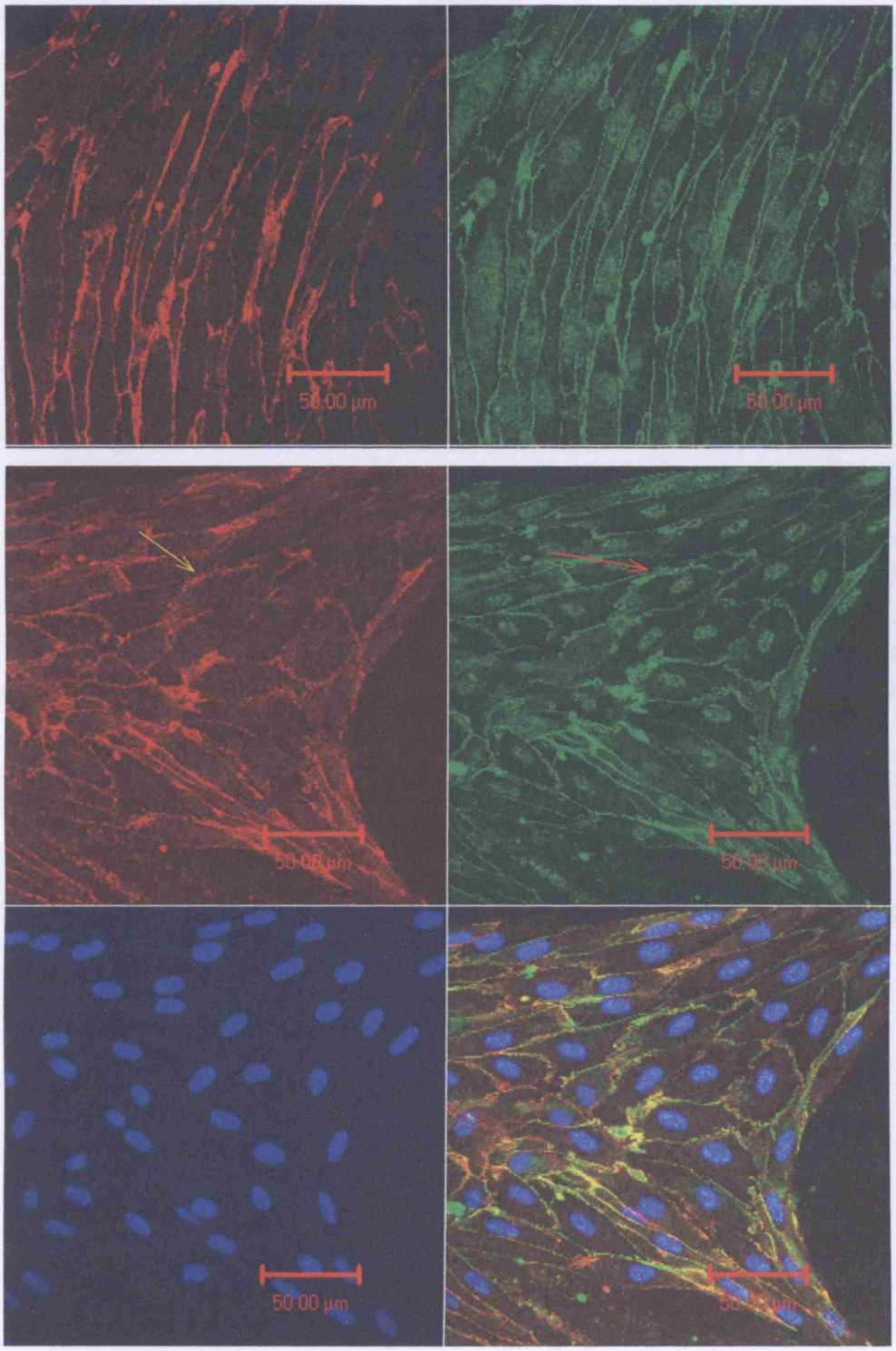


Fig 3.54. 100 ng/ml VEGF-A and primary retinal endothelial cells. At 100ng/ml of VEGF-A induces some fragmentation of junctional staining.

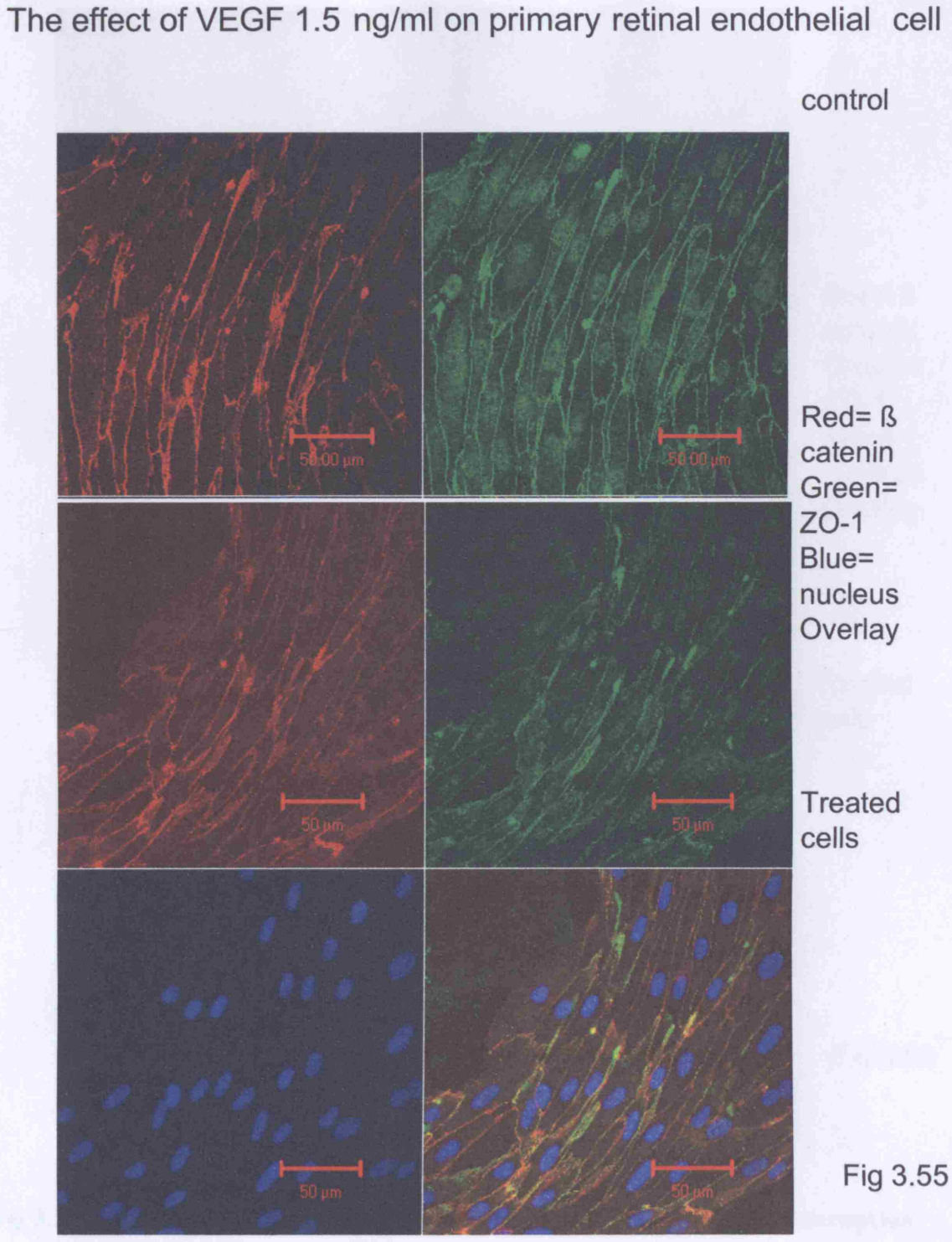


Fig 3.55. 1.5ng/ml VEGF-A and primary cultures. There was minimal disruption of junctional staining at this concentration of VEGF-A

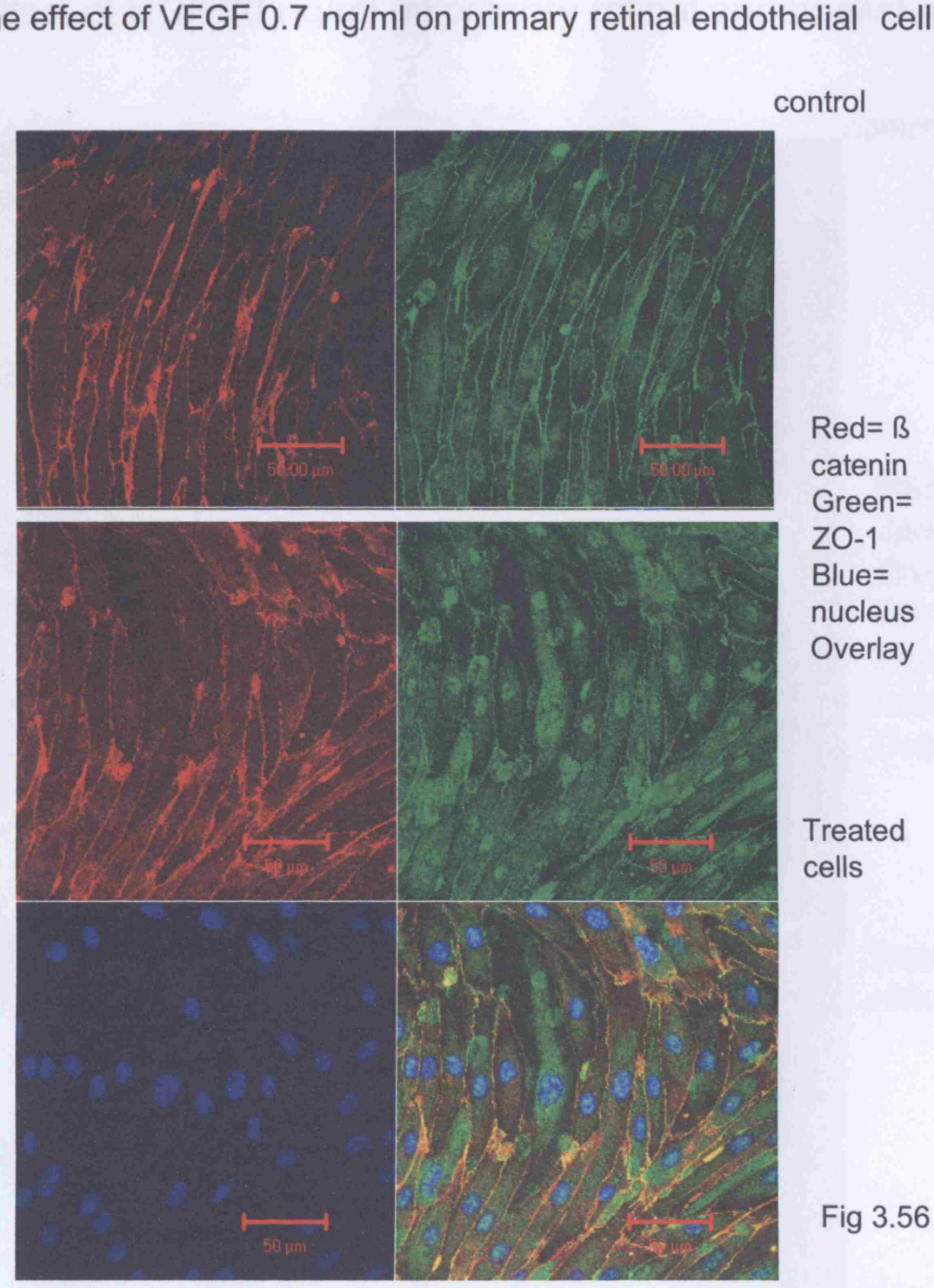


Fig 3.56. 0.7ng/ml VEGF-A and primary cultures. There was minimal disruption of junctional staining at this concentration of VEGF-A.

The effect of HGF 20 ng/ml on primary retinal endothelial cell

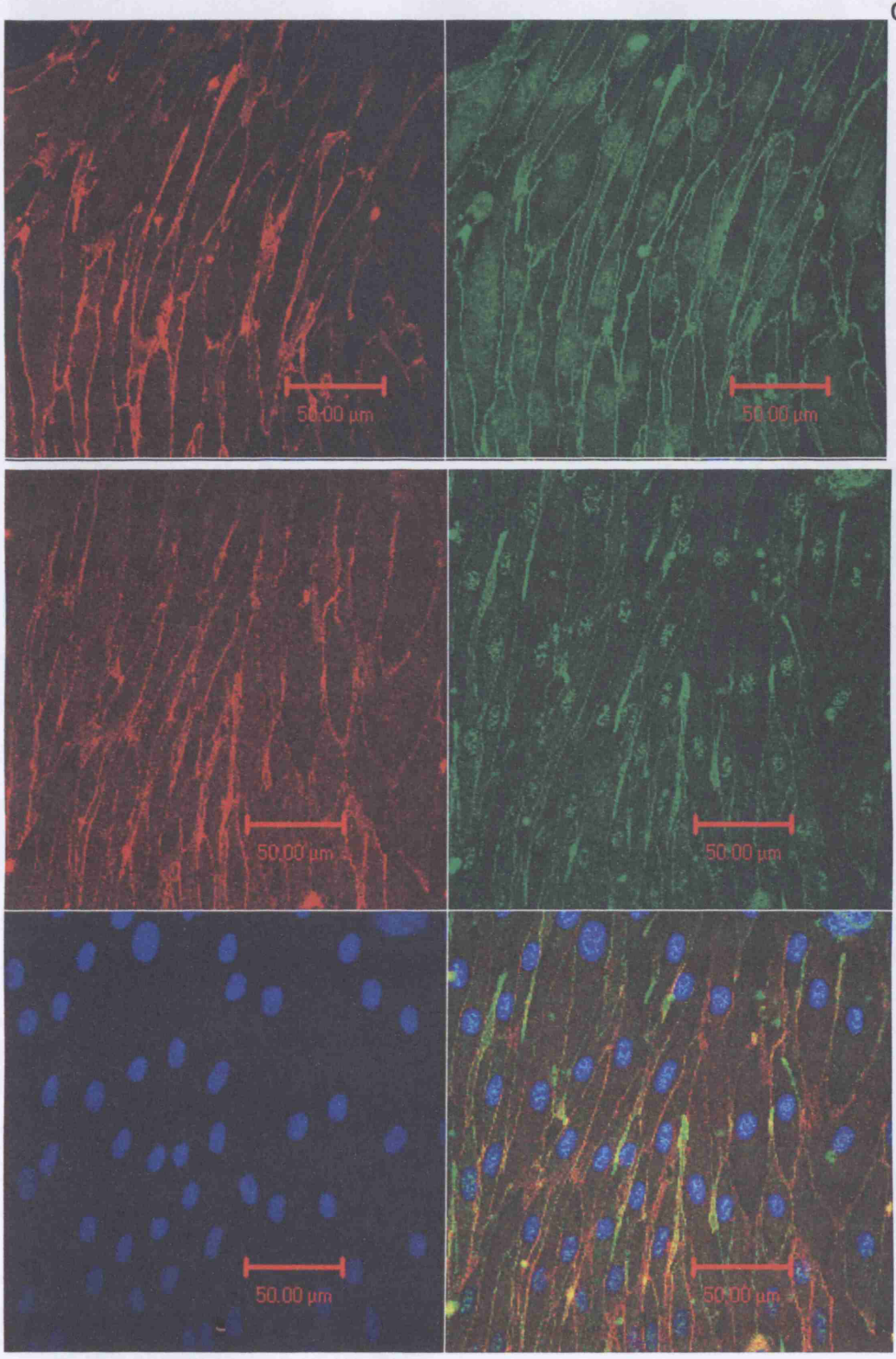


Fig 3.57

Fig 3.57. Primary cultures and 20ng/ml HGF. Minimal junctional change is seen at this concentration of HGF.

The effect of HGF 1.5 ng/ml on primary retinal endothelial cell

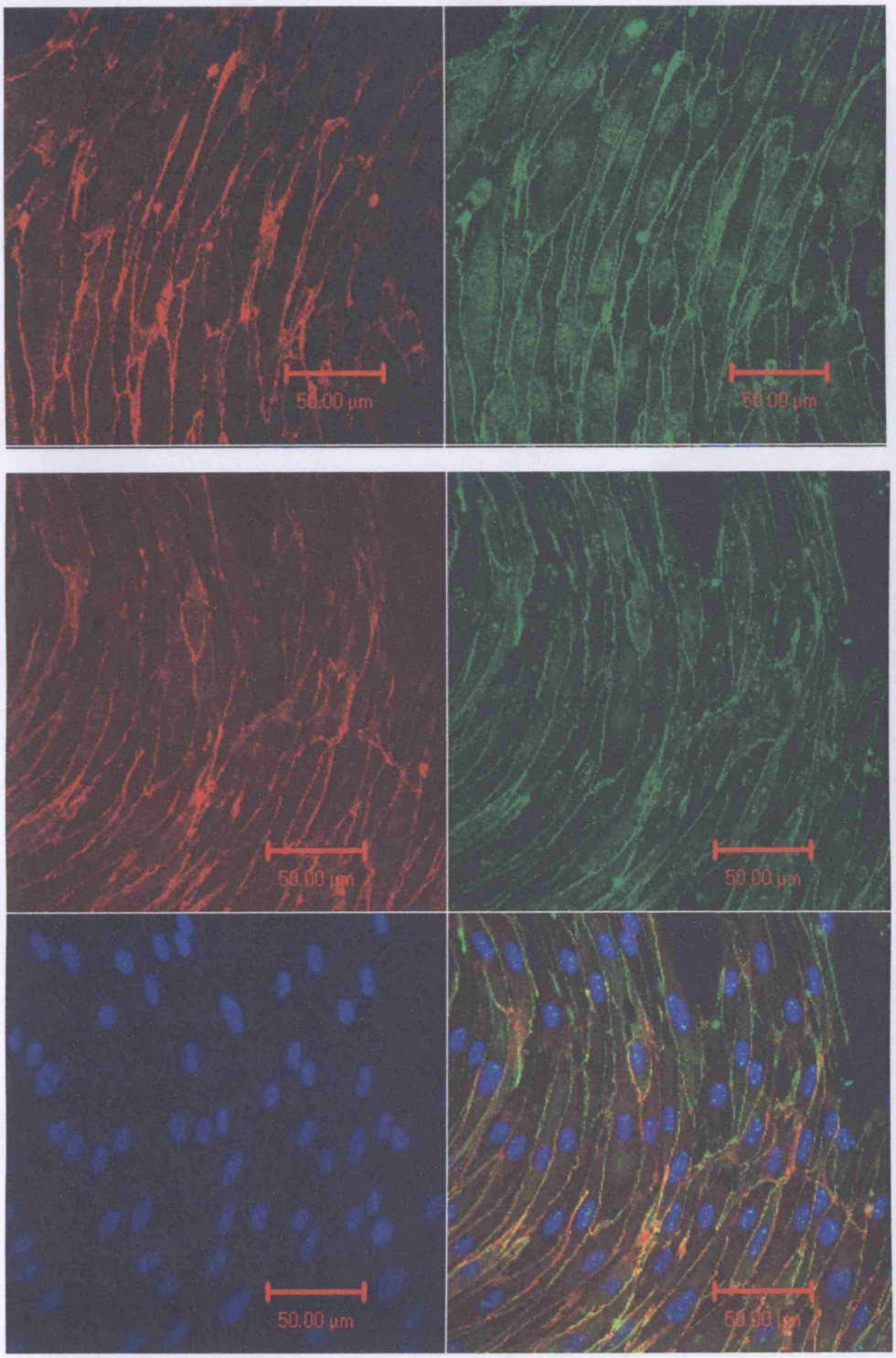


Fig 3.58

Fig 3.58. Primary cultures and 1.5ng/ml HGF. Minimal junctional change is seen at this concentration of HGF.

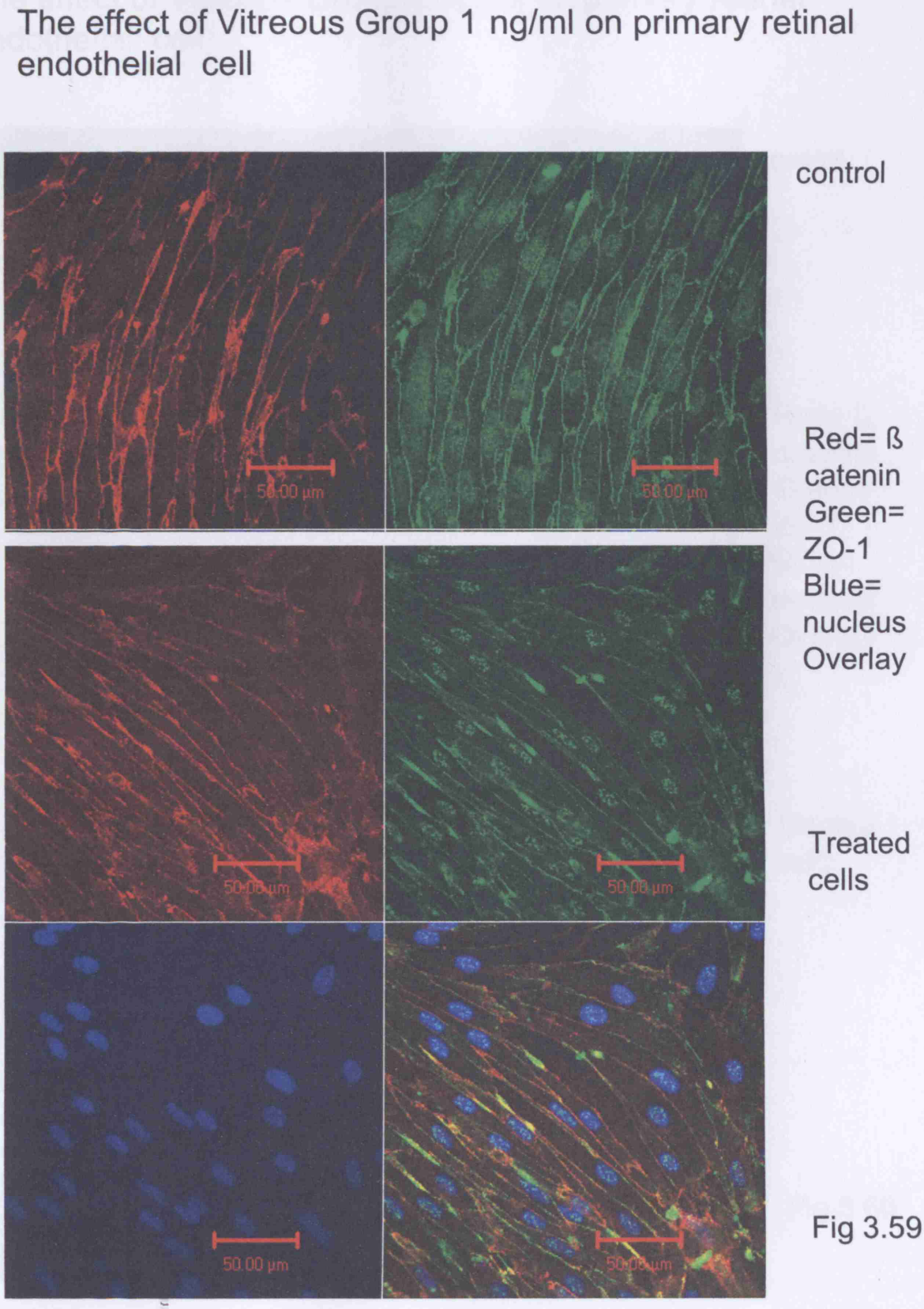


Fig 3.59. Group 1 vitreous and primary cultures. Minimal junctional change is seen with vitreous from Group 1.

The effect of Vitreous Group 2 ng/ml on primary retinal endothelial cell

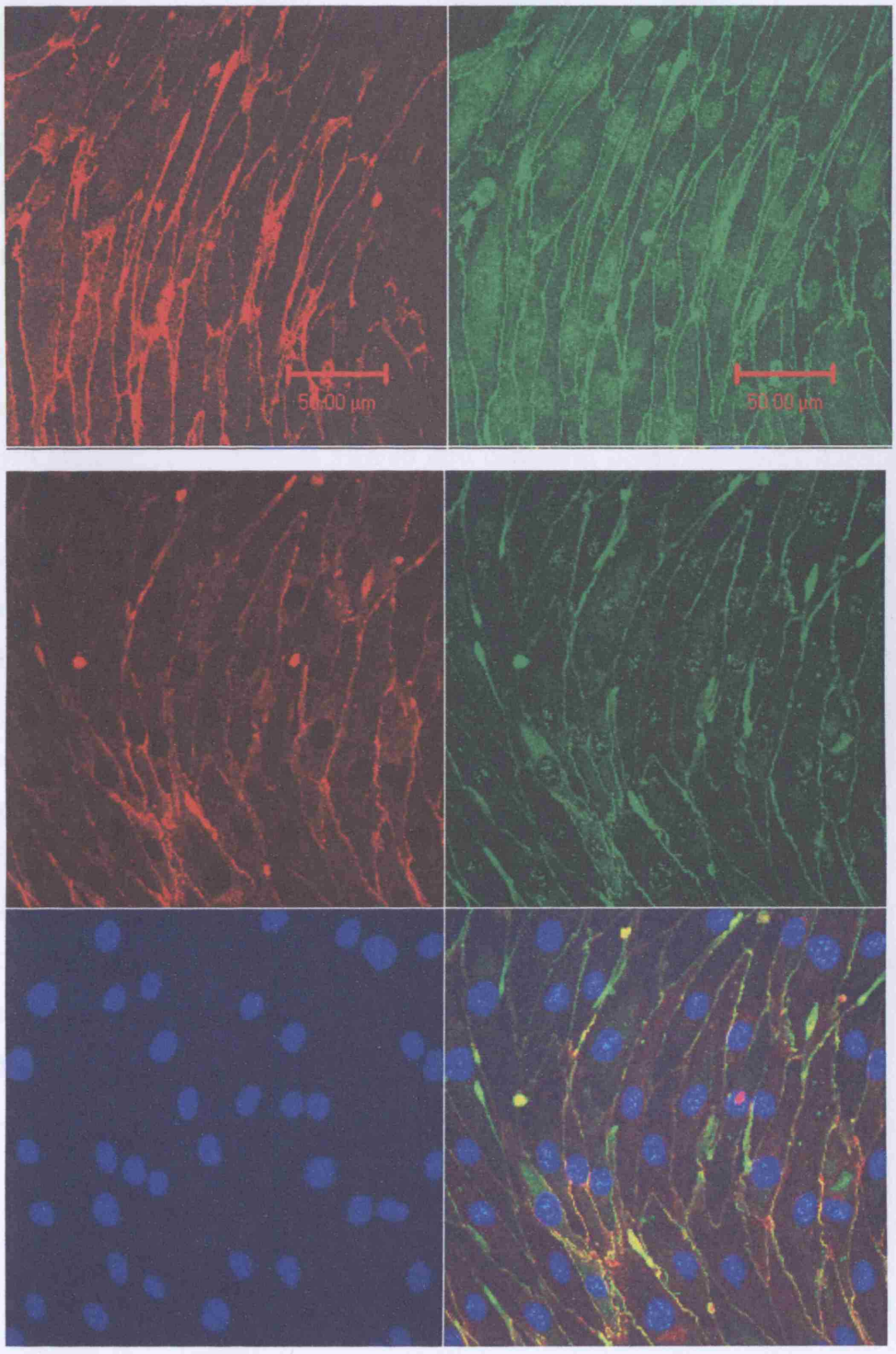


Fig 3.60

Fig 3.60. Vitreous Group 2 and primary cultures. Minimal junctional change is seen with vitreous from Group 2.

Negative control (secondary antibody alone) staining for ZO-1 and b-catenin is seen in Figure 3.48

Chapter 3.3.1.3 PERMEABILITY STUDIES

Using FITC 40 and FITC 150, the permeability of the JG2 cells was measured as degree of fluorescence per millilitre after VEGF-A 100ng/ml treatment at varying time points and compared to control.

Confluent monolayers of JG2 cells were less permeable compared to the inherent permeability of the transwell pores (**Figure 3.61**). This would suggest a degree of tight junction formation had formed into confluent layers to impede the paracellular flow of FITC 40 and 150. However when cells were pre treated with VEGF-A for varying time points up to 24 hours prior to the addition of FITC 40 and 150 and the subsequent fluorescence / ml (as a measure of permeability) measured up to an hour after FITC addition, there was no substantive difference seen in the permeability when comparing the different VEGF-A treatment times for either of the FITC-dextran weights used (**Figure 3.62**). The absolute values of the fluorescence / ml was different between the two FITC-dextran weights which could just reflect the effect of different sizes (weight) of the FITC-dextran, more than any VEGF-A induced functional increase in junctional permeability because this difference was consistent through all VEGF-A pre-treatment time points. These results would suggest that in using these cells, in this system, we were unable to discriminate functional effects of VEGF-A treatment.

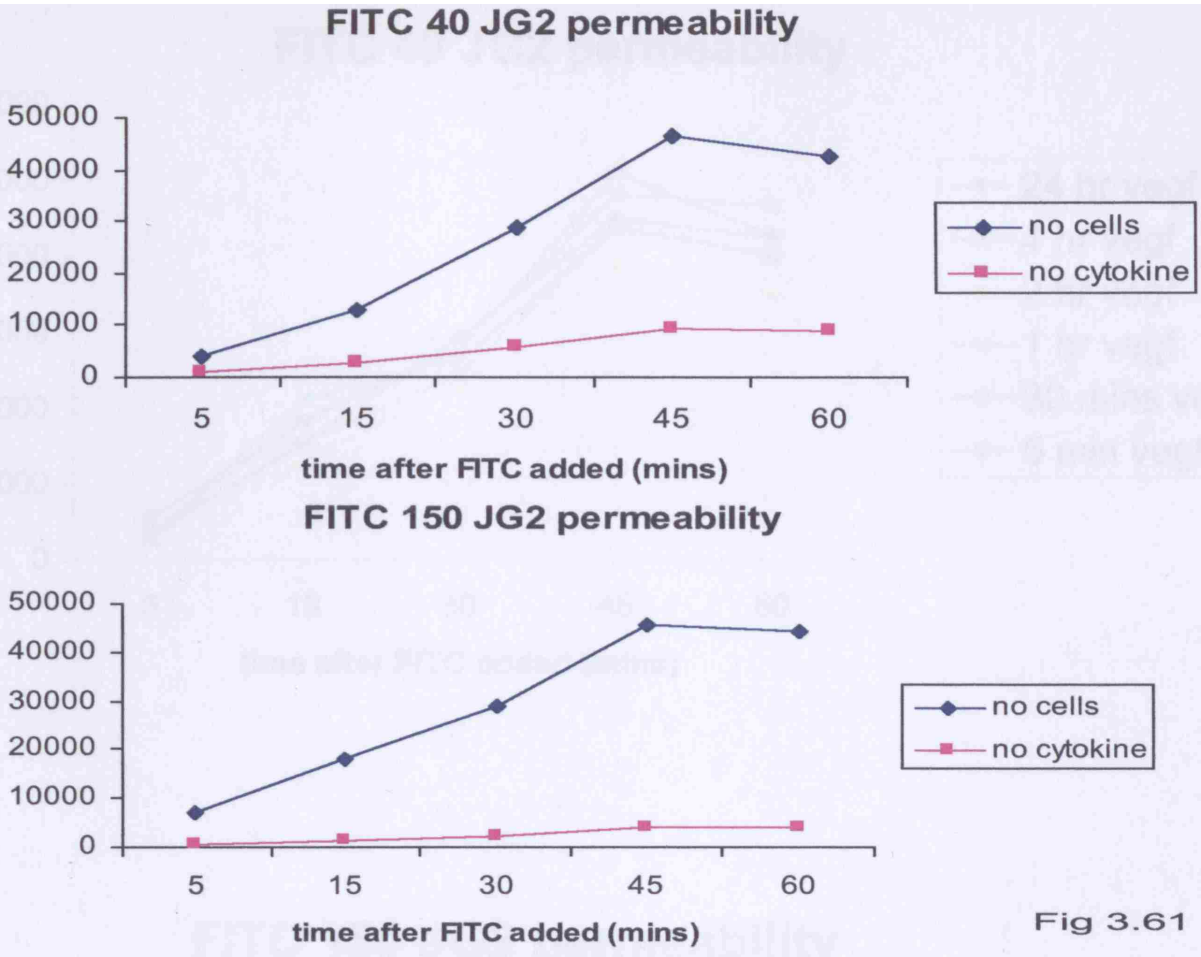
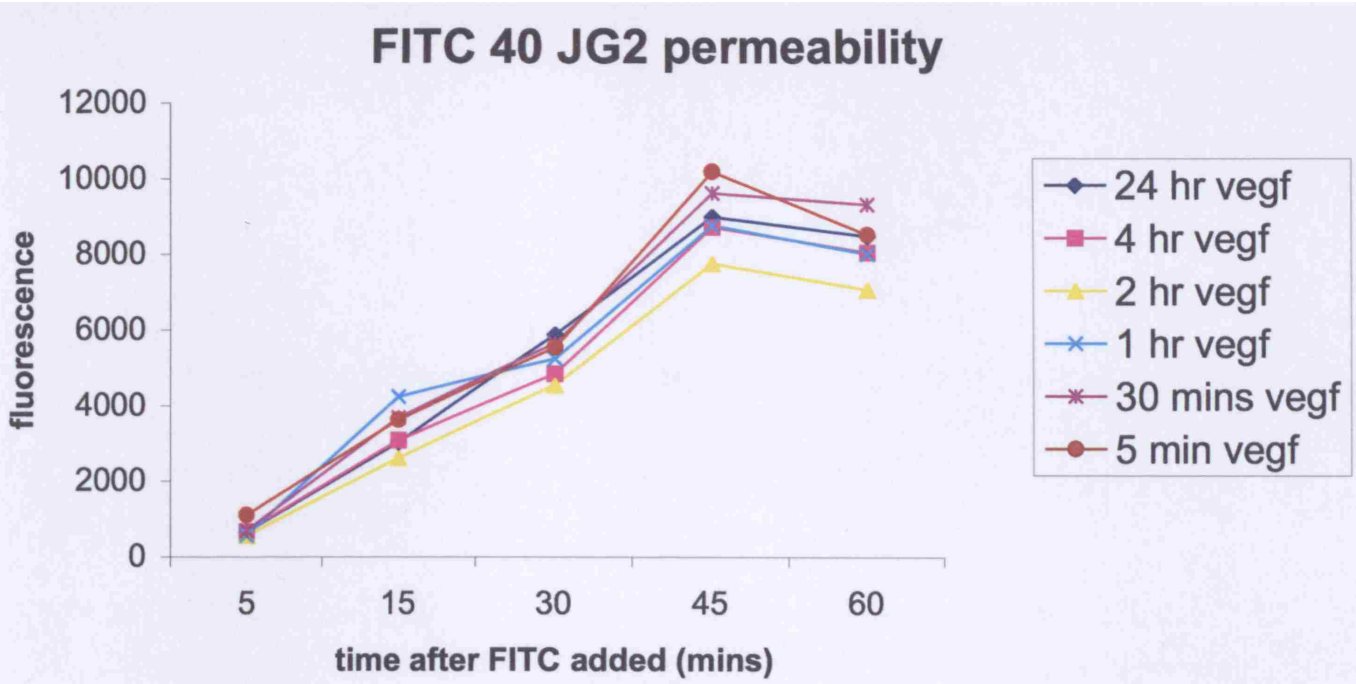


Fig 3.61. Baseline fluorescence of JG2 retinal endothelial cells.

Confluent non-treated JG2 cells (no cytokine) were less permeable than the inherent permeability of the transwell pores, indicating confluency.



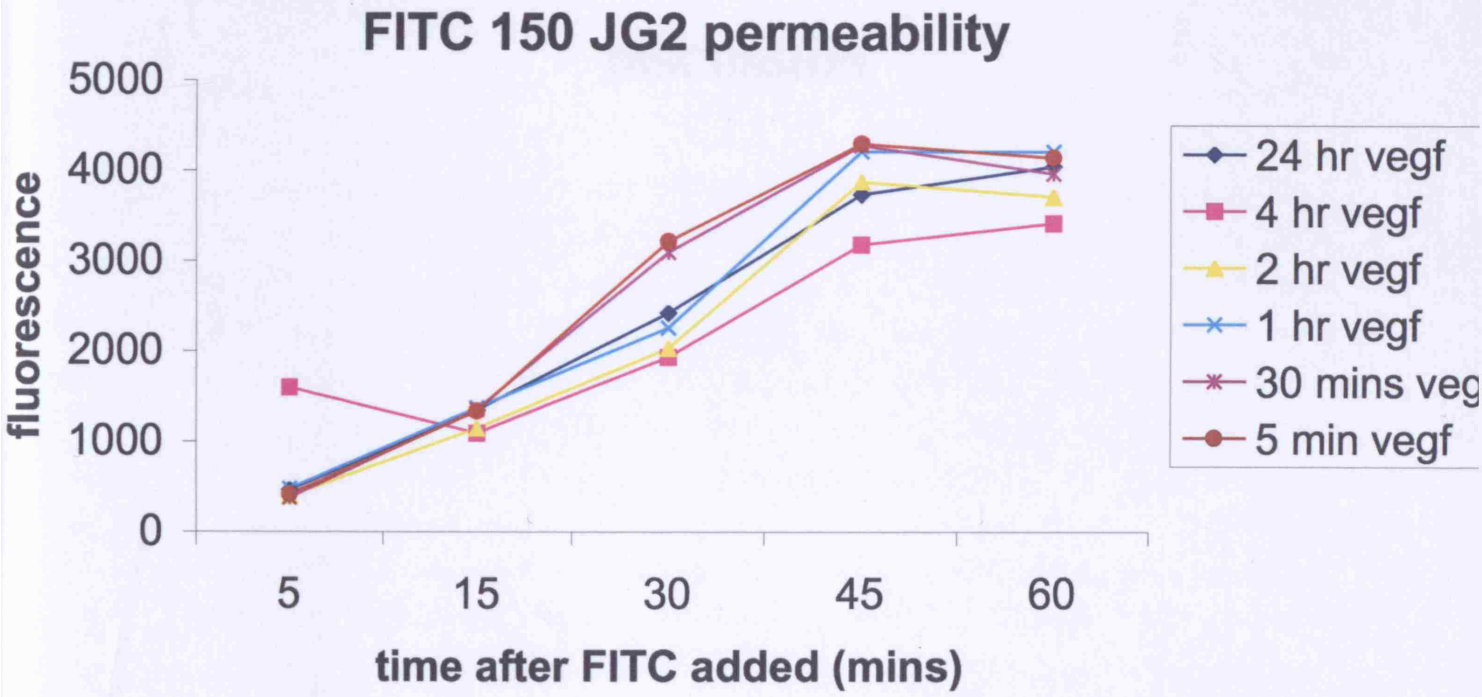


Fig 3.62. FITC-dextran fluorescence post VEGF treatment in JG2 cells.

No difference in the fluorescence /ml after the addition of FITC-dextran between the different VEGF treatment times. However there was a difference in the absolute values of fluorescence/ml between the different FITC-dextran weights.

CHAPTER 4

DISCUSSION

CHAPTER 4.1 GENERAL DISCUSSION OVERVIEW: STRENGTHS AND WEAKNESSES

This is the first work to correlate structural and morphological macular oedema appearance with the cytokine concentration in the vitreous and to describe how this morphology changes after pars plana vitrectomy in relation to the changes of the cytokine concentration.

We were able to describe two distinct diabetic macular oedema morphologies and demonstrate different baseline VEGF concentrations in them that may have reflected a different aetiology in their development. We also showed that there is a difference in the structural response post vitrectomy between these two groups which again may reflect the differences in the VEGF concentration post vitrectomy. This is the first report correlating macular oedema structural appearance as judged on OCT with baseline and post-vitrectomy VEGF concentrations.

We described the angiopoietin (angiopoietin 1 and 2) concentrations in diabetic retinopathy and interestingly in diabetic macular oedema the angiopoietin 1 concentration was half that of angiopoietin 2 and also in two patients which showed differing responses post vitrectomy (one showing an improvement in the foveal thickness, the other the opposite response) there was difference in the angiopoietin concentrations post-vitrectomy in these two patients which may also suggest that not only is there the change in VEGF concentration but also that of the angiopoietins which may determine the response of vitrectomy on diabetic macular oedema combined with the effect of release of any vitreomacular traction.

Other cytokines were also present in the diabetic vitreous of patients with macular oedema in significant concentrations. These were HGF, TGF β, MMP 9 and endothelin-1. HGF is also angiogenic and TGF b and MMP 9 can also contribute to the disruption of the tight junctions.

The anti-angiogenic profile was also disrupted in a probable gradual manner. Initially there seemed a significant decrease in the soluble Flt-1 receptor concentration in diabetic macular oedema and then in proliferative disease PEDF also significantly decreases.

Clinically the trial demonstrated an effect of pars plana vitrectomy with all the trials

conducted under consistent and strict procedures particularly visual acuity measurements (which were by a masked observer and non-biased) and none of the patients post vitrectomy underwent cataract extraction so all visual acuity results published are not confounded by the effect of cataract removal. A non-masked observer took OCT measurements but an internal fixation device was used to maintain consistency of image acquisition from fixation and the internal automated software was used to measure the foveal thickness and macular volume.

The cell culture work demonstrated that the immortalized cell line was not an effective or sensitive enough to use for the study of the effects of cytokines on cell junctions.

The principal weaknesses of the study were the small numbers of patients used in the trials and in particular the postoperative aqueous samples that were used to assess VEGF concentrations post vitrectomy. Also in the RCT there were too few patients particularly after the high drop-out rate. Also some of the cytokines assays used had high intra - and inter- observer coefficient of variations suggesting a less sensitive assay even though it was at or just below 21% e.g. Il-1Ra.

SPECIFIC DISCUSSION FOR CLINICAL AND LABORATORY RESULTS

CHAPTER 4.2 CLINICAL STUDIES

The vitreous is considered to contribute to the pathogenesis of diabetic macular oedema (DMO). Hikichi evaluated the association between the natural history of diabetic macular oedema and the state of the vitreous in 82 eyes over a 6-month period and their findings suggested that macular oedema was more likely to resolve in the presence of vitreomacular separation (Hikichi *et al.* 1997). Lewis was first to describe a positive effect of pars plana vitrectomy (PPV) on diabetic patients who had a thickened and taut posterior hyaloid membrane with traction on the macula. There were improvements in both the vision and resolution of macular oedema (Lewis *et al.* 1992) and the flattening of the shallow macular detachment which develops secondary to the tractional forces (Lewis *et al.* 1992; Van Effenterre 1993) (Harbour *et al.* 1996). Therefore tangential vitreomacular tractional forces (Lewis *et al.* 1992)

combined with the local presence of a number of cytokines and growth factors (Kent *et al.* 2000) are thought to contribute to the development of DMO.

In this prospective study of 12 eyes of 12 patients with persisting fovea-involving diabetic CSMO, two groups of patients were defined with distinct OCT macular profiles. In Group 1 there is a dome-shaped thickening with the posterior vitreous cortex partially detached over the perifoveal area, but remaining focally attached at the fovea. In Group 2 there is a diffuse thickening of the macula with no signs of a focal posterior hyaloid separation. For Group 1 the foveal thickness was twice and macular volume 1.5 times the similar indices for Group 2 at baseline. Massin *et al.* have also described similar macular profiles in their patients with diabetic CSMO (Massin *et al.* 2003). Giovannini also showed two similar profiles on OCT in 18 diabetic patients with CSMO (Giovannini *et al.* 2000). The reason for the two different patterns was not clear but, based on cytokine levels, differences in the vitreomacular tractional forces between the two groups may account for the OCT arrangements seen. The dome-shaped profile with its focal attachment of the posterior vitreous cortex on the fovea and adjacent perifoveal detachment could develop from greater tractional forces exerted on the macula and therefore a more thickened fovea and a larger macular volume compared to the other group with its diffusely thickened macula with no similar arrangement of the posterior vitreous cortex. It was not possible to tell either on biomicroscopy or on FFA which configuration would be observed on OCT.

Following PPV, there was improvement in the macular profiles and in the structural macular indices for all 12 patients and also for both groups. Collectively there was a 26% decrease in the foveal thickness and an 18% decrease in the macular volume for all 12 patients 12 months after surgery. However for Group 1, the foveal thickness decreased by 33% and the macular volume by 32% during the same post-surgical period compared to baseline. For Group 2 there was only a 9 % decrease in both these indices for the same time period post surgery. These changes may reflect the differences in the vitreal tractional forces exerted on the macula and may suggest that in those eyes where such forces play a significant contribution to the pathogenesis of DMO, the removal of these forces by PPV may lead to reductions in retinal thickness especially in the early postoperative period: in Group 1 both these structural indices

demonstrated large decreases in the first 2 weeks following surgery compared to Group 2 which showed increases for both these parameters. OCT could therefore be used to select diabetic macular oedema patients for surgery.

Following the structural improvement in the macula and fovea there was also functional improvement in both the visual acuity and perifoveal cone thresholds 12 months after surgery. This was despite the central CSMO persisting on average for 15 months (range 12-18 months) prior to surgery and all had been previously treated with macular photocoagulation. Perifoveal cone thresholds showed improvement for both groups compared to pre operative levels. However on correlating threshold and retinal thickness, changes in similar areas revealed that two thirds of patients showed improvement in both these indices in the macular field. Both the visual acuity and the perifoveal cone thresholds improved by 10%.

Relief of the vitreomacular tractional forces and the removal of permeability-inducing cytokines at the vitreomacular interface after vitrectomy provide potential mechanisms which would allow for decreases in the foveal thickness and improvement in vision post surgery. In a report of inflammatory-induced macular oedema, removal of the vitreous containing inflammatory mediators resulted in improvements in visual acuity (Dugel *et al.* 1992). Furthermore it has recently been suggested that another potential mechanism of action is an increase in oxygenation of the inner retina as a result of PPV (Stefansson 2001). The increased oxygenation would allow for a decrease in VEGF secretion, thus removing a potent vasopermeability inducing cytokine (Senger *et al.* 1983).

To date there have been a number of studies evaluating the role of PPV in patients with CSMO and an attached non-thickened hyaloid face (Tachi and Ogino 1996) (Ikeda T 1999) (Amino and Tanihara 2002; Giovannini *et al.* 2000; Ikeda *et al.* 1999; Sato *et al.* 2002). The majority of these studies were retrospective, and hence without an evident standardised visual acuity testing protocol by a masked observer. Even though our study represents the data of a small number of patients, the VA testing was performed by a standardised format by a masked observer.

In all these studies, including this one, there was improvement in visual function.

Tachi and Ogino (1996) reported that 53% of 58 eyes treated improved by 1 line of Snellen acuity. Tachi reported similar levels of improvement in 85 eyes. However 66% to 74% of the eyes undergoing PPV in these 2 studies also underwent phacoemulsification at the same time as the PPV. The studies do mention the structural benefits in PPV with the resolution of macular oedema as judged clinically by FFA. In a prospective study of 13 eyes undergoing PPV without phacoemulsification by Otani and Kishi, 38% (5/13) improved by 2 lines of vision. They also performed OCT assessment of the changes and noted that in those patients where the foveal thickness decreased by more than 50% of the preoperative measurement there was a gain of two lines of vision (Otani and Kishi 2000). However, in their recent prospective study of 14 eyes in 7 patients, the foveal thickness significantly decreased over the 6 month follow up period, but the visual acuity showed no such significant improvement over the same time period (Otani and Kishi 2002). The authors suggest that a longer follow up is needed to evaluate the efficacy of vitrectomy for DMO. In the current series, only at 12 months did the foveal thickness and therefore the visual acuity achieve significant improvement.

Previous macular laser treatment may affect the overall degree of visual improvement post surgery. Heij *et al.* (2001) reported in their retrospective series of 21 eyes that 7 eyes *without* preoperative macular photocoagulation achieved a median percentage improvement of 77% in vision, whilst 12 eyes with preoperative macular photocoagulation only achieved 15% improvement in vision, suggesting a significantly better visual outcome in eyes that had undergone no previous macular photocoagulation In the current series all patients had undergone previous macular laser treatment and the percentage improvement in vision was 20%.

It has been shown that in patients with non-proliferative diabetic retinopathy and CSMO, the foveal cone amplitudes are subnormal and the implicit times are slower compared to normal eyes (Weiner *et al.* 1997). In eyes with diabetic macular oedema, there is diminished directional sensitivity and diminished visual pigment density in photoreceptors located in the fovea (Lardenoye *et al.* 2000). Directional sensitivity determines the alignment of the photoreceptors to absorb parallel rays of light, which is more visually efficient than bundles of light entering the photoreceptor obliquely. Therefore reduced directional sensitivity would allow for oblique bundles of light to

enter the photoreceptor and result in poorer vision. This reduced directionality is thought to be due to changes in cone alignment in early macular oedema, but with the persistence of the oedema, secondary changes in the structure and / or loss of photoreceptors account for the loss of directional sensitivity. Visual pigment density reflects photoreceptor function. In eyes with resolved macular oedema there is also reduced pigment density secondary to the loss of photoreceptors and change in photoreceptor orientation (Lardenoye *et al.* 2000). Macular photocoagulation has been shown to worsen photoreceptor implicit timings and decreases in amplitudes on multifocal ERG (electroretinogram) (Greenstein *et al.* 2000). Therefore these findings suggest that in CSMO there are structural and functional changes in the foveal and macular photoreceptors, which affect the level of vision and in which the destructive effect of macular photocoagulation may worsen. In these patients the improvement in visual acuity probably reflects changes to the structure and alignment of the photoreceptors resulting from the decrease in the foveal thickness and macular volume. Larger improvement in the functional indices may not have been possible because of the changes induced in the photopigment density caused by the longstanding oedema and the multiple laser burns that are not reversible despite improvements in the macular volume and the foveal thickness induced by PPV.

In the literature, there is debate as to the location of the fluid in macular oedema. Histopathological studies suggest that the fluid maybe intracellular in the Muller cell, which undergoes swelling and degeneration that leads to the cystic spaces (Fine and Brucker 1981). Other studies have suggested that the fluid is extracellular and that Muller cell changes and neuronal loss are secondary events (Tso 1982). In the dome-shaped group there was a large central pocket of fluid within the thickened retina whilst for the second group there was retinal thickening with reduced intraretinal reflectivity and multiple intraretinal cysts. These OCT images whilst not conclusively determining the site of fluid accumulation within the retina, would suggest a possibility of a greater extent of extracellular fluid in the dome-shaped group, which after the release of traction has the greater propensity of resorption. This seems to begin within 2 weeks of surgery. However, for the diffuse-low elevation group, the multiple intraretinal cysts could relate to swollen or degenerated Müller cells, in which instance limited improvements in macular volume would be seen post surgery, as is the case. For this group the small improvement in macular volume and foveal

thickness maybe related to the removal of the cytokines and mediators of vascular permeability deposited within the vitreous.

In summary this clinical study showed that PPV can lead to structural and functional improvements in patients with persistent CSMO. We also showed that there are 2 distinct groups of patients with fovea-involving CSMO based on the macular profile as imaged by OCT. This could reflect and confirm the involvement of vitreous traction upon the macula as a component of the pathogenesis of macula oedema.

To further evaluate the role of traction we extended the clinical study to ascertain the effect of peeling the inner limiting membrane (ILM) in addition to vitrectomy and comparing whether ILM peeling provides a greater structural and functional improvement of the macula against simply removing the vitreous solely.

It has previously been reported that a posterior precortical vitreous pocket exists anterior to the posterior retina extending to the temporal vascular arcades (Kishi and Shimizu 1990). The posterior surface of this pocket is attached to the internal limiting membrane, so that after inducing a posterior vitreous detachment during vitrectomy only the anterior surface of the pocket is removed leaving the posterior surface in situ (Kishi *et al.* 1986). The posterior surface would then continue to exert tractional forces upon the retina. Therefore vitrectomy combined with ILM peel would also result in resolution of macular oedema (Gandofer *et al.* 2000). However this study was retrospective involving 12 eyes with 10 of the eyes having a taut and thickened hyaloid face. Therefore clinical improvement in either the appearance of the macula could be attributed to removal of this taut hyaloid face rather than to the ILM alone. Removal of the ILM would allow for mobilization of the retina with subsequent release of tangential traction (Rice *et al.* 1999).

In this prospective, comparative study, the role of vitrectomy with ILM peel was explored in a representative cohort of patients with persisting fovea-involving diffuse diabetic CSMO who clinically (and confirmed intra-operatively) showed no signs of a taut posterior hyaloid face. We were also able to compare the results from this cohort against the results of vitrectomy alone in a second cohort of patients recruited with similar criteria and similar OCT macula profiles. The macular volume in the ILM peel

group (Group 2) at 12 months showed significant improvement (p=0.039) despite the other structural (foveal thickness) and functional (vision) parameters showing improvement during the post operative time period for both groups of patients. On comparing the two groups, the results suggest no functional advantage in ILM peeling despite a structural advantage (Group 1).

The ILM represents the structural boundary between the retina and the vitreous with the collagenous vitreous cortex attached to one side and the Müller cell end-feet to the other. A recent study examined the ultrastructural effects of ILM peel in two cadaveric eyes. The findings demonstrate damage and swelling of the Müller cell end-feet and their adherent inner processes which run towards to the inner nuclear layer not only within the peeled area but also extending to the transition zone of the non-peeled area which was adjacent to the peeled area(Wolf *et al.* 2004). The removal or damage of the end-feet may cause severe changes in the physiological function of the Müller cell which functions, in particularly within the region of the end-feet, as a site of intense exchange of retinal waste products (e.g. potassium ions and protons) with the vitreous body acting as a buffering sink (Newman and Reichenbach 1996). Such biochemical changes in particular the removal of potassium ion may affect the electrophysiological output of the retina as the Müller cell is thought to be involved in the generation of the b wave in the electroretinogram (ERG). Using focal macular ERGs in eyes after removal of the ILM during macular hole surgery there was a selective delay of recovery in the ERG b-wave 6 months after surgery suggestive of a change in the normal Müller cell physiology in the macular region (Terasaki *et al.* 2001). This alteration in physiology may explain the lack of a significant improvement in vision despite a decrease in the structural indices after surgery. In addition all patients in the current study had repeated grid photocoagulation before surgery that almost certainly affected visual function. The pattern ERG decreases after macular laser treatment and patients on average had 3 macular laser treatments suggesting a compromised macular function at the onset. Such electrophysiological changes may explain no significant improvement in vision in the peel group 12 months after surgery despite having a significantly better baseline vision compared to the no peel group.

The greater structural improvement after ILM peel may result from the additional

removal of residual vitreous cortex after the initial surgical posterior vitreous separation, as suggested in a recent study which showed that after surgical posterior vitreous separation residual vitreous cortex is still seen (Sonoda *et al.* 2004). The residual vitreous cortex may contribute to continued tractional forces upon the macula and the consequent development of oedema.

Both these clinical studies suggest and confirm the presence of tractional forces, both antero-posterior and tangential, acting on the macula extending from the vitreous. The macula returns towards a normal anatomy in relation to structural parameters, but never returns to a completely normal anatomy with complete resolution of oedema. This is reflected by the lack of a vastly significant improvement in function (both visual acuity and cone function) despite the structural improvement. The 10% functional improvement seen after vitrectomy alone may reflect structural and functional changes already present in the photoreceptors secondary to the oedema and macular laser. The limited functional improvement after ILM peel may again reflect the chronic nature of the oedema (at least 12 months prior to surgery) and the possibility of ultrastructural damage to the Muller cell and its retinal processes after ILM peel.

The last of the clinical studies conducted was to conduct a randomised pilot study to test the methodology and sample size needed to carry out a larger definitive randomised control trial to evaluate the role to PPV against standard macular laser treatment.

The findings from this pilot trial suggest no advantage of PPV over conventional ETDRS laser in the management of diabetic macular oedema. However we also demonstrated a stabilization of ETDRS vision in four of the PPV patients with improvement in the vision in one of them. The PPV group showed a greater improvement in the perifoveal cone function over the 12 month follow-up period.

A combination of vasopermeability-inducing cytokines as well as vitreo-macular tractional forces is thought to promote the development of diabetic macular oedema. Alleviation of such forces by PPV would allow for resolution of macular oedema (Lewis *et al.* 1992). The patients included in the trial all had persistent clinically

significant macular oedema present for at least 12 months in spite of prior laser photocoagulation with an average of 3 macular laser treatments. The OCT macular profiles at baseline suggested only one patient in each group having had the dome-shaped macular configuration with partial PVD and vitreo-foveal attachment. It is conceivable that tractional forces in such cases may significantly contribute to the pathogenesis of macular oedema. The others had the diffuse-low elevation profile where no such vitreomacular relationship was seen on OCT.

Four patients from both groups showed structural and functional improvement 12 months after initial treatment. One patient belonged to the vitrectomy group whilst the other three, belonging to the laser group, developed spontaneous vitreous detachment three months after laser treatment.

This suggests that separation of the posterior vitreous cortex from the macular surface either by vitrectomy or as a spontaneous event may allow for structural and functional improvement, which is variable. This variability may reflect the continuing actions of the vasopermeability-inducing cytokines produced in diabetes and / or possible damage to the macular surface by the peeling of the posterior hyaloid. The histological examination of membranes harvested at vitrectomy from patients with vitreomacular traction syndrome revealed large segments of the ILM (Shinoda *et al.* 2000). This is in contrast to plasmin-induced vitreoretinal separation, which resemble more closely spontaneous PVD, no such damage to the ILM was seen (Gandofer *et al.* 2000). Damage to the ILM may change Muller cell physiology and so alter electrophysiological recovery as tested by ERG (Terasaki *et al.* 2001), as explained earlier. The less traumatic spontaneous PVD, which occurred in three patients in the laser arm, may have contributed to the thinning of the macula and to the visual improvement and thus narrowed the difference in the success rates for each treatment arm at 12 months. Also macular function as measured electrophysiologically is compromised in diabetic patients with macular oedema after focal laser treatment (Greenstein *et al.* 2000) (Lardenoye *et al.* 2000) (Greenstein *et al.* 2000). This compromise seems to involve the loss of foveal cones (Lardenoye *et al.* 2000), oedema-induced changes in Muller cells (Yamamoto *et al.* 2001) (Fine and Brucker 1981), as well as the associated damage of non-treated areas of adjacent retina (Greenstein *et al.* 2000). In the current series, the patients were recruited after having

undergone previous macular laser treatment (an average of 3 times) with the oedema present for an average of 14 months prior to treatment in each arm. Both the high number of laser treatments and the relatively long duration of oedema may have contributed to the limited improvement in foveal cone function.

However, the improvement after vitrectomy may have also been in part due to the removal of the vasopermeability-inducing growth factors contained in the vitreous gel (e.g VEGF-A) and the improved oxygenation of the retina post-vitrectomy (Stefansson 2001) which would lead to decreased VEGF production.

The results of this pilot trial suggest little difference between the two treatments i.e. that the proportion of eyes with a successful visual acuity outcome after laser photocoagulation was 4 % greater than after PPV (75% versus 71%). This percentage success rate is an estimate based on the patients seen at the 12 months follow-up time point. Two patients in the laser group and one in the vitrectomy group failed attendance at this final time point and so no assumption was made as to whether they were successful or a failure in terms of ETDRS vision. The aim of the study was to determine the numbers needed for a definitive study in this setting. Given the small numbers and the drop-out rate (as high as 25%), the following calculations can be made: to detect (at 5% significance) a relative improvement of 4 % at 12 months would require approximately 4,647 patients per treatment arm (9,294 total) (90% power). Given this small difference between the treatment groups, the very large numbers needed for a definitive randomised trial, and that our data revealed a small but significant improvement in vision in the laser treated patients, such a study may prove to be too much of an organisational challenge as it would require participation of a large number of centres.

SUMMARY OF CLINICAL STUDIES

These studies confirmed the contribution of vitreomacular traction in the pathogenesis of diabetic macular oedema, but also suggested that this contribution is only partially responsible for its development. The other contributors to the formation of macular oedema are likely to be the cocktail of cytokines and growth factors in the vitreous.

CHAPTER 4.3 GROWTH FACTOR STUDIES

An imbalance of angiogenic factors and inhibitors may underlie the progression of diabetic retinopathy. In this study, the level of these factors has been evaluated in a group of diabetic patients whose retinopathy varied from moderate non-proliferative to proliferative. The structural patterns in patients with persistent diffuse clinically significant macular oedema (CSMO) were also correlated with the changes in these parameters after surgery. Such post vitrectomy correlations, together with the levels of these factors, may explain the varying response of surgery seen in these patients but also provide insight into the molecular mechanisms of diabetic macular oedema *in vivo*.

Two important angiogenic stimulators are VEGF-A and HGF. The vitreous VEGF-A concentrations were significantly raised in the diabetic patients compared to the control macular hole patients, but there was no difference in concentration between nonproliferative diabetic retinopathy (NPDR) and active PDR. Similarly, HGF levels were significantly raised in the diabetic patients compared to the control group. Both VEGF-A and HGF are mitogenic for endothelial cells (Ferrara 2000), (Cai *et al.* 2000) and induce changes in retinal vascular permeability (Qaum *et al.* 2001) (Aiello *et al.* 2004). Therefore, the results for both these factors confirm their up-regulation in the diabetic state allowing them to alter the retinal vascular bed to induce permeability and neovascularization. The greater concentration in NPDR compared to quiescent retinopathy or FTMH may represent active vascular remodelling sites in NPDR compared to quiescent or FTMH with the greater VEGF-A concentration stimulating these sites. However, despite significant VEGF-A up-regulation compared to quiescent disease or FTMH, no such difference in VEGF-A concentration between active proliferative or NPDR disease was seen, even though both clinical entities demonstrate active pathological vascular changes, but significantly different clinical pictures. This paradox in the concentration of VEGF-A may relate to the changes in the anti-angiogenic agents, soluble flt-1 receptor antibody (sFlt-1) and PEDF. HGF was found in equally high concentrations in both NPDR and PDR also providing an angiogenic stimulus in both clinical phenotypes.

Alternate splicing of VEGF receptor 1 mRNA results in production of sFlt-1, an

inhibitor of VEGF signalling which acts by sequestering VEGF and possibly forming dimers with full-length receptors resulting in dominant-negative effects (He *et al.* 1999; Shibuya 2001). In a mouse model sFlt-1 can suppress VEGF-induced retinal vascular permeability (Gehlbach *et al.* 2003). PEDF provides the principal anti-angiogenic activity of human vitreous (Dawson *et al.* 1999). These anti-angiogenic growth factors showed a decrease in their levels in the diabetic state. In the patients with nonproliferative retinopathy and macular oedema, the sFlt-1 levels decreased compared to macular hole patients, but the PEDF levels were similar to the macular hole patients. This would suggest that the fall in the sequestering effect of sFlt-1 would allow VEGF-A and HGF to influence the endothelial cells sufficiently to induce permeability, but as PEDF concentrations are sufficiently high, the angiogenic potential of these factors would be limited. However in PDR as the sFlt-1 concentrations fall further, combined with a significant decrease in PEDF, VEGF-A and HGF are then able to produce the angiogenesis seen in PDR.

Although study numbers were limited, OCT macular profiling revealed two distinct groups of patients with fovea-involving CSMO. Vitreal growth factor and protein analysis were as different in these two groups as was their structural profile. There was the dome-shaped group in a fifth of the patients (Group 1) and a diffuse-low elevation group accounting for the remaining patients (Group 2). Similar macular profiles have previously been identified in patients with diabetic CSMO (Massin *et al.* 2003) (Giovannini *et al.* 2000). Similar to our findings, they described a dome-shaped group with a thickened retina, signs of vitreomacular traction and a large central cyst. In a second group there was mild retinal thickening with reduced intra-retinal reflectivity and multiple intra-retinal cysts. The difference in the anatomical profiles between the two groups would suggest greater vitreomacular tractional forces contributing to macular oedema in the dome-shaped group with the posterior hyaloid cortex attached to the fovea. A taut posterior hyaloid face has been described in a subgroup of diabetic patients with CSMO (Lewis *et al.* 1992) and OCT examination in this subgroup confirmed partial posterior hyaloid separation with traction exerted upon the macula and increased retinal thickness (Kaiser *et al.* 2001) similar to the configuration and retinal thickness seen in the dome-shaped (Group 1) patients. Vitreo-tractional forces affect retinal cohesiveness decreasing the retinal tissue pressure or increasing compliance leading to fluid accumulation and increased retinal

thickness (Lobo *et al.* 1999). A predominant mechanical pathogenesis for this clinical profile seems likely as the baseline vitreous and aqueous levels of VEGF-A was lower compared to Group 2 (the diffuse-low elevation group).

The higher protein levels in Group 2 (diffuse-low elevation) suggest a greater degree of blood retinal breakdown and vascular permeability in these patients compared to Group 1 (dome-shaped). Vitreous VEGF-A levels were higher in Group 2 and VEGF-A can disrupt tight junctions of the blood retinal barrier (Antonetti *et al.* 1998) allowing the leakage of protein and the accumulation of extracellular fluid. Previous reports have correlated the aqueous VEGF-A concentrations with the severity of diabetic macular oedema implicating VEGF-A in the breakdown of the blood retinal barrier (Funatsu *et al.* 2002). In a similar study of 20 diabetic patients with macular oedema and retinopathy ranging from mild to severe non-proliferative, the average vitreous VEGF-A concentration was 861 pg/ml, which is in broad agreement with the current study results (Funatsu *et al.* 2002). Both these studies are therefore consistent with the current findings. However, it was possible to explore this relationship further in the current study by correlating structural with molecular parameters pre- and post-surgery to demonstrate that despite these molecular differences, there was anatomical thickening of the macula in both groups with patients at similar stages of diabetic retinopathy. This would suggest that both VEGF-A -induced and mechanical vitreotractional forces contribute by varying degrees to the pathogenesis of macular oedema. These tractional forces may exceed fluid accumulation in the dome-shaped group because the growth factor levels were lower in this group compared to the other group.

Post-vitrectomy aqueous VEGF-A concentrations after PPV and the relationship with macular volume changes also revealed further differences. In Group 1, VEGF-A increased whilst the macular volume decreased with the largest fall in volume occurring in the first two weeks after PPV, but by 6 weeks there was no significant difference in the volume changes in Group 1 compared to baseline. This change could reflect a release of vitreomacular tractional forces allowing for macular compression despite increased VEGF-A concentrations. Alleviation of the tractional forces could induce a rapid difference in the biochemical output profile of the Müller cell via the internal limiting membrane to which the cytoplasmic footplates of this cell are

attached. It has been proposed that VEGF-A is produced within several areas of the Müller cell and subsequently transported to the inner retina to its target cell, the endothelium (Amin *et al.* 1997). Mechanical stretch and shear forces induce VEGF-A release from endothelial and other retinal cells and RPE cells respond to pulsatile stretch by an increase in VEGF-A secretion (Seko *et al.* 1999), where such tractional forces are transmitted to the RPE via an extracellular sheath surrounding photoreceptors which acts as a physical bridge between the neurosensory retina and the RPE (Hollyfield *et al.* 1989). Therefore Müller and RPE cells may respond to fluctuations in the surrounding growth factor profile or tractional forces by changing the level of VEGF-A synthesis or transport to the inner retina. In contrast, the VEGF-A aqueous levels decreased in Group 2, as did the macular volume, reaching significant improvement at 6 weeks post-surgery. This improvement in the macular volume occurring secondary to a decrease in VEGF-A levels may provide the molecular explanation for the variable response seen in the macular volume in some patients after this treatment. Post-operative aqueous VEGF-A could reflect vitreous levels in the same way that baseline aqueous reflect baseline vitreous levels (Aiello *et al.* 1994). Retinal ischaemia correlated with aqueous VEGF-A in patients with ischaemic Central Retinal Vein Occlusion (iCRVO) (Boyd *et al.* 2002), suggesting that measured VEGF-A in the aqueous reflects local pathology and retinal VEGF-A, which may diffuse forwards. Furthermore, recently Funatsu *et al.* demonstrated correlation between vitreous and aqueous VEGF-A in diabetic patients (Funatsu *et al.* 2005). These studies, together with our work, support the suggestion that cytokines and growth factors within the vitreous diffuse towards the aqueous. Therefore the aqueous concentrations of cytokines and growth factors would reflect the changes seen in the vitreous. The vitreous concentrations themselves would probably reflect the retinal microenvironment, which is difficult to assess directly.

Also this continuing production of VEGF-A post-vitrectomy as estimated by the concentrations of aqueous VEGF-A in our patients would suggest that despite vitrectomy, the underlying diabetic process which stimulates retinal VEGF-A production still continues. This continued production post-surgery and the level of production and how it interacts with the vitreomacular changes brought about by surgical intervention ultimately defines the effect of vitrectomy as seen in our two groups of patients.

TGF β1 acts as a powerful fibrogenic agent stimulating matrix synthesis (Border and Noble 1994). Furthermore the diabetic vitreous also undergoes pathological cross-linking and non-enzymatic glycation which affect the collagen structure (Sebag *et al.* 1992). A combination of both processes may alter the integrity of the posterior cortical vitreous by changing it and inducing macular traction especially in Group 1, which has the higher TGF β1 levels. Vitreomacular traction induces a shallow macular detachment, which is relieved by PPV with subsequent improvement in visual acuity (Lewis 2001). Lastly Muller cells are known to produce TGF β1. Recently, in a cell culture model, Müller cells have been shown to release TGF β1 onto neighbouring endothelial cells which then release matrix metalloproteinase 9 (MMP-9) (Behzadian *et al.* 2001). MMP-9 levels were increased in both groups, in one group possibly contributing to the biochemical changes in the vitreous with TGF β1 to produce the vitreo-macular relationship seen in Group 1 and in the second group possibly contributing to increased permeability though induced changes in endothelial tight junctions (Harkness *et al.* 2000).

The data from our current study confirm that the balance between angiogenic agents (VEGF-A and HGF) and the anti-angiogenic factors (sFlt1 and PEDF) is disturbed in the diabetic state and suggest that the extent of the change in these anti-angiogenic agents may determine the retinal phenotype seen as both angiogenic agents are up-regulated. Two structural sub-types of diabetic macular oedema can be described. The first is characterised by posterior hyaloid traction and an increased macular volume, which does decrease after PPV irrespective of VEGF-A levels, suggesting that primary tractional forces are important in its aetiology. Raised TGF β1 in this group may stimulate a fibrotic response in the posterior hyaloid providing the mechanism for generating tractional forces. The second subtype is characterised by a combination of diffuse macular thickening and an elevated VEGF-A level, both of which decrease after PPV indicating that VEGF-A maybe important in the aetiology in this group. Equally, raised HGF levels in both groups would additionally provide further insult to increase permeability.

VEGF-A is a vasopermeability-inducing agent with known disruptive effects on

retinal endothelial tight junctions (Antonetti *et al.* 1999). However, animal and *in vitro* models have shown that VEGF does not work alone. VEGF-A has been shown to work in concert with the angiopoietins, (Asahara *et al.* 1998) which interact with the Tie-2 Receptor. To date only concentrations of VEGF-A in the ocular fluids of diabetic patients are known (Aiello *et al.* 1994).

This is the first report of the concentration of angiopoietins in the vitreous of diabetic patients. In PDR, the vitreous angiopoietin 1 level was similar to those of macular hole (FTMH) patients. The angiopoietin 2 concentration was below the sensitivity of the assay for both FTMH and PDR. This finding reflects the role of angiopoietin 2 in facilitating the process of angiogenesis at sites of active remodelling (Maisonpierre *et al.* 1997). Therefore if there is no active angiogenesis (as in FTMH), its level would not be elevated. Our results in PDR vitreous samples appear to contradict the role of angiopoietin 2 as a facilitator of angiogenesis, but this may simply reflect the fact that in long-standing PDR, there is little active vessel replication. Angiopoietin 1 promotes stabilization of immature neo-vascularization by promoting the recruitment of supporting cells (Suri *et al.* 1996). In established PDR, at the time of vitrectomy, new vessels are mature with limited active angiogenesis and therefore low concentrations of the angiopoietins would be expected. In FTMH, no active neovascular processes are known to occur, explaining the minimal concentration of angiopoietin 1. In fact, FTMH patients may represent the normal non-neovascular vitreo-retinal environment where angiopoietin 1 is at a basal level that may help promote and maintain endothelial integrity.

In contrast, patients with NPDR had angiopoietin 1 vitreous concentrations that were approximately half those of angiopoietin 2. In our series, patients had at least moderate and five cases had severe NPDR. From clinical observation there is sufficient retinal ischaemia and capillary closure in such cases with the formation of IRMA (intraretinal microvascular abnormalities - precursors of new vessels). This represents the point in the natural history of diabetic retinopathy where early neovascularization occurs. Such dynamic changes within the retina are probably reflected by the increased angiopoietin 2 concentration combined with a relative change in the angiopoietin 1 to angiopoietin 2 ratio allowing angiopoietin 2 to predominate at the Tie 2 receptor. The predominance of angiopoietin 2 at the Tie 2

receptor would promote increased vascular permeability in combination with elevated VEGF in NPDR (Murata *et al.* 1995) thus facilitating breakdown of the blood retinal barrier.

Interestingly, our findings in two patients who had intra- and two post-operative aqueous samples concur with the role of angiopoietin 2 as a pro-permeability agent. In the one patient, in whom the foveal thickness had decreased at 3 months after surgery, the angiopoietin 1 concentration had progressively increased during the initial 6-week period following surgery. We postulate that in this case, the anti-permeability effect of angiopoietin 1 acted to reduce the amount of macular oedema as measured by OCT. In the second patient in which the post-operative macular oedema increased up to 3 months, there was a concomitant reduction in angiopoietin 1 aqueous concentration leading to increased permeability and macular oedema mediated by angiopoietin 2 (probably with VEGF-A). It seems that the morphological effects of the early changes in angiopoietin 1 after surgery may be borne out clinically some weeks later. If this model of angiopoietin 1 function is correct, then an increase in angiopoietin 1 in this situation would precipitate a cascade of intracellular events which would eventually lead to the 'tightening' of the blood retinal barrier (Gamble *et al.* 2000). However a larger study correlating macular volume, as measured by OCT, and post-operative aqueous angiopoietin concentrations would be required to confirm these findings.

The retinal microcirculation undergoes changes even before the onset of clinical disease. In type 1 diabetes there is a glycaemic-control related increase in capillary pressure and flow, resulting in injury to the endothelial cell, which triggers an increase in extracellular matrix production. This increase in extracellular matrix production leads to basement membrane thickening and disturbance of autoregulation (Tooke 1986). In type 2 diabetes, there is a disturbance in microvascular vasodilatory capacity. In both situations this leads to an increase in capillary perfusion (Sandeman *et al.* 1990). According to Starling's principal, an increase in capillary pressure would allow for increased passage of fluid and macromolecules into the retinal interstitium.

The results of the current study demonstrate that in diabetes there are changes in both the haemodynamic-related (e.g. endothelin-1) and inflammatory and anti-inflammatory factors.

Endothelin-1 showed significant decreases in non-proliferative retinopathy compared to controls with a subsequent significant increase in the proliferative retinopathy. The fall in endothelin-1 in NPDR would reflect the reported increase in retinal blood flow seen in NPDR (Tooke 1986) as its vasoconstrictive effect would be minimal. Also in non-proliferative patients with macular oedema, these results suggest endothelin-1 positively correlated with both the foveal thickness and macular volume. Endothelin-1 can interact with endothelin A and B 1 and 2 receptors. Endothelin A and B 2 receptors mediate vasoconstriction, the B 1 receptor mediates vasodilatation via the release of nitric oxide (Rubanyi and Polokoff 1994). The correlation of macular structural indices of oedema with endothelin-1 in the non-proliferative retinopathy patients could be related to the activation of ET-B1 receptor producing vasodilatation whilst in PDR patients, who have the greater levels of endothelin-1, allowing for a vasoconstrictive effect via the activation of both endothelin receptors A and B 2. Raised levels of endothelin-1 have also been reported in the vitreous of PDR patients (Oku *et al.* 2001).

As NO is possibly an important autoregulatory factor in the control of vascular tone in the retina, we investigated indirectly for its presence in the vitreal samples. Previous reports have shown increased levels of nitrate and nitrite (nitric oxide) in PDR (Oku *et al.* 2001) (Hattenbach *et al.* 2000) (Yilmaz *et al.* 2000). Our results suggest diabetic patients showed no significant differences from controls in nitric oxide levels, even though the diabetic patients did have lower levels compared to controls. These differences in results may be related to the method of analysing NO levels. Our method used an ELISA kit detecting total nitrite levels as an azo dye product of the Greiss reaction. Nitrite is produced from nitrate which itself is the product of conversion of nitric oxide with oxygen. Others have measured nitrate or nitrite levels in vitreous or aqueous using liquid chromatography or spectrophotometry. These differences may reflect the difficulty in measuring this short acting molecule and relying on indirect measurements of its subsequent metabolites to reflect its concentration. Despite this discrepancy in the results of NO (ours lowers, previous reports higher), it still suggests that there is an abnormal change in its concentration in diabetic retinopathy, altering the vasoregulatory capacity of retinal vessels. However it may not be the sole mediator for altering or producing the reduced retinal flow seen in PDR.

The proinflammatory cytokine Il-1 β has been previously shown to be raised in the vitreous of proliferative diabetic retinopathy (Yuuki *et al.* 2001) and in the retina of diabetic retinas (Carmo *et al.* 1999). In our study IL-1 β levels was raised in PDR patients in agreement with these findings but in NPDR and in controls, the levels were below that of sensitivity of the assay. This may suggest minimal inflammatory activity in the early stages of retinopathy but as the disease progresses with significant capillary loss and retinal ischaemia (PDR), inflammation begins to increase. Indeed the anti-inflammatory cytokine IL-1Ra was significantly higher in the control vitreous compared to the diabetic vitreous, which may allow for the unopposed action of IL-1 β especially in PDR. IL-1 β can induce the apoptosis of retinal endothelial cells in culture which is inhibited by addition of IL-1 Ra (Kowluru and Odenbach 2004). IL-1 β can also induce leukocyte recruitment to the retina (Bamforth *et al.* 1997) and may thus contribute to leukostasis. Despite the observation that in both NPDR and PDR IL-1Ra levels were similar, the anti-inflammatory environment of NPDR is maintained purely because the pro-inflammatory response is minimal at this stage of the disease process, despite leukostasis having been shown to occur from the early stages in an animal model of the diabetic retina (Miyamoto and Ogura 1999). IL-1 β can recruit white cells (Hanai *et al.* 1995), usually through a mechanism of increased cell adhesion. However, a recent report has suggested that even in the presence of leukostasis impacting within multiple retinal capillaries during these early stages, retinal blood flow is not affected (Abiko *et al.* 2003). Therefore the gradual increase in leukostasis is unlikely to produce the decrease in retinal blood flow seen in PDR. Leukostasis seems to develop secondary to endothelial dysfunction induced by elevated free fatty acids produced as a metabolic consequence of insulin resistance (Abiko *et al.* 2003). As disease progresses a greater level of leukostasis may be seen as endothelial dysfunction increases throughout the retina. This would not only cause a reduction in blood flow, but would allow for the activated leukocytes to elaborate a host of inflammatory cytokines e.g. IL-1β, which is seen at high levels in PDR; in NPDR the concentration of IL-1β is at a minimal concentration which is unable to overcome the inhibitory effect of IL-1Ra.

Prostacyclin (PGI2) is a vasoactive prostaglandin produced by vascular cells that can induce vasodilatation *in vivo* at physiological concentrations. PGI2 can induce

vascular permeability and mediate NO-induced ocular vasorelaxation (Dodge *et al.* 1991). Thus changes in prostacyclin concentrations may alter retinal haemodynamics. The prostacyclin concentration reflected NO concentrations. In diabetic patients the levels were lower than in controls, but not significantly so. Reports have suggested that NO can stimulate prostacyclin production by the endothelial cell (Davidge *et al.* 1995) and our results for both factors may reflect this finding. These mechanisms would suggest a decreased vasodilatory effect in diabetes, but the changing concentrations of endothelin-1 may override the influence of prostacyclin-NO axis of vasodilatation.

This is the first report demonstrating the levels of endothelin-1 in non-proliferative and proliferative diabetic eye disease. The results suggest a fall in endothelin-1 in NPDR reflecting the increase in retinal blood flow in NPDR with a subsequent decrease in PDR, which reflects the attenuation of blood flow in PDR. Furthermore, this study also shows changes in the markers of inflammation and anti-inflammation where the anti-inflammatory environment in diabetes is reduced compared to controls.

SUMMARY OF CYTOKINE AND GROWTH FACTOR FINDINGS (**Fig 4.1**)

These findings describe the molecular profile of cytokines and growth factors seen in diffuse diabetic macular oedema. The various cytokines and growth factors fall broadly into four groups: 1) the pro-angiogenic agents (e.g. VEGF-A, HGF, angiopoietin 2, 2) anti-angiogenic / antipermeability agents (e.g. soluble flt-1 receptor antibody, PEDF and angiopoietin 1 and 3) supporting cytokines which can either act to modulate or act as an effector agent (e.g. MMP 9 as a effector, TGF β1 as a modulator) and 4) haemodynamic and inflammation-related cytokines (Prostacyclin, NO, Endothelin-1, Il-1β, Il-1 Ra).

These different molecules directly affect or influence the development of macular oedema. The pro-angiogenic agents directly damage the blood retinal barrier and together with modulating factors, influence the vitreous matrix, by possibly changing the mechanical relationship of the posterior vitreous and macula. The combination of these events would act as the primary catalyst for the development of macular

oedema. VEGF induces a transient, immediate but reversible hyperpermeability in cultured endothelial cells, lasting up to 30 minutes and probably secondary to an increase in caveolin-coated trascytotic vesicles (Feng *et al.* 1999). Subsequently there begins a sustained increase in paracellular permeability starting 4 to 6 hours after VEGF-A treatment. VEGF-A induces the activation of the urokinase system and its receptor (uPAR) (Behzadian *et al.* 2003). HGF can also activate the uPAR system (Hall *et al.* 2004). This would initiate plasmin formation with subsequent disruption of endothelial intercellular attachments by uPAR-mediated initiation of proteolytic activity (Mandriota *et al.* 1995). Combined with the proteolytic activity of uPAR, MMP 9 also acts in a similar manner to disrupt retinal endothelial cell tight junctions (Harkness *et al.* 2000). Therefore, both angiogenic agents (which were increased in the vitreous) are active together with two important proteolytic systems (MMP 9 levels were also increased in the diabetic vitreous), providing the impetus for the breakdown of the blood retinal barrier.

Superimposed upon this primary mechanism would be the influence of the anti-angiogenic factors. The decreasing levels of the antiangiogenic cytokines in the diabetic retina / vitreous would then allow for the angiogenic cytokines to act without antagonism.

This imbalance in molecular homeostasis within the retina, the degree of this imbalance, and the level of influence of an altered mechanical vitreous may determine the macular profile and pathology in individual patients. In addition to this disturbed homeostasis there exists the background influence of haemodynamics (changes in retinal blood flow, capillary hydrostatic pressure and related cytokines) and inflammatory related (leukostasis with capillary closure) processes occurring within the retina.

Pars plana vitrectomy would remove the mechanical effect of the vitreous and to a certain extent, temporarily, also remove the cytokine cocktail, though as the diabetic process is continuous, the underlying imbalance in the molecular profiles would re-emerge, but also be altered by the mechanical removal of the posterior vitreous face as seen in post vitrectomy cytokine profiles which correlate with clinical parameters.

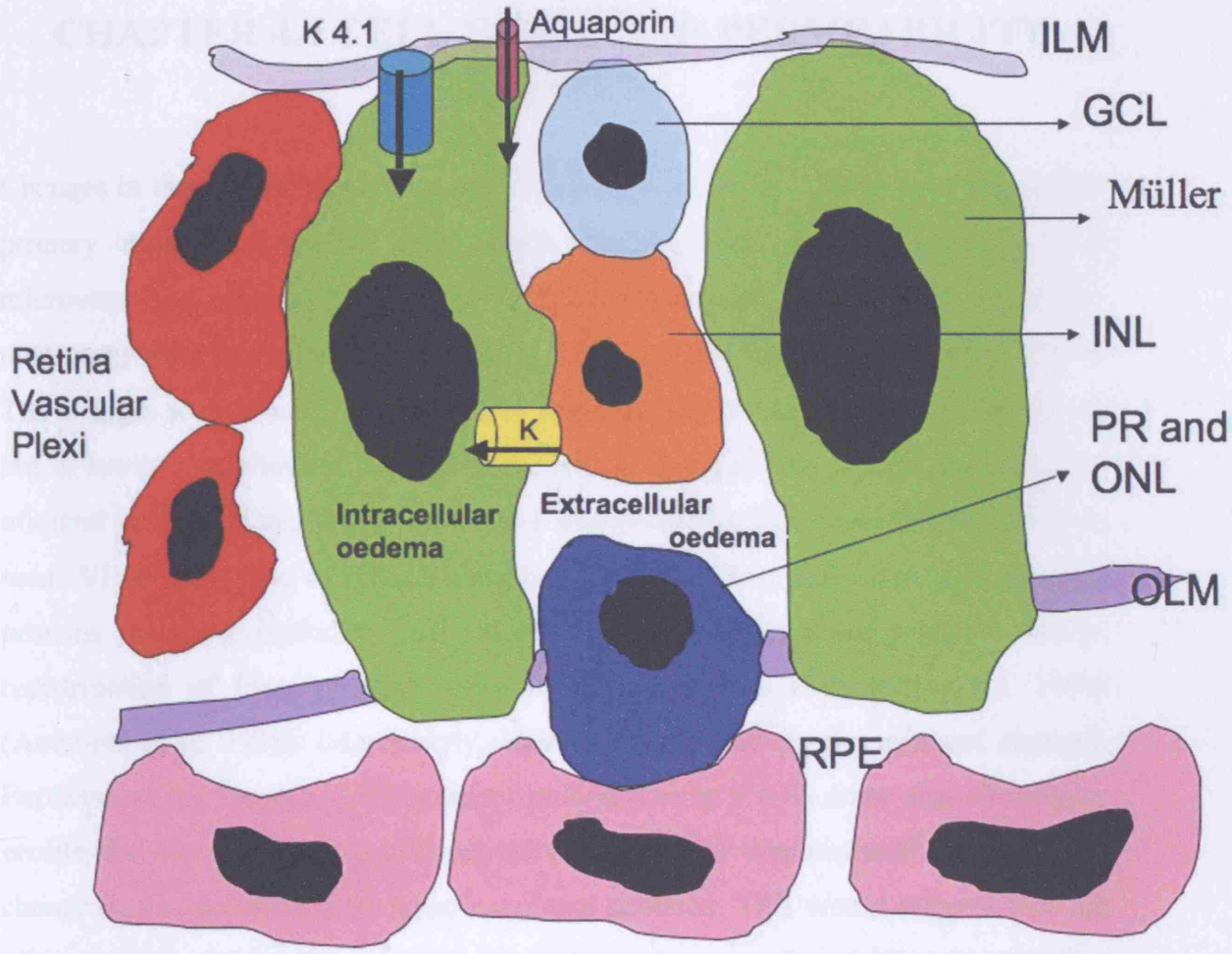


Fig 4.1. Potential Pathways of Oedema. The schematic describes the potential pathways of intracellular oedema (involving potassium channels) and extracellular oedema involving breakdown of the endothelial retinal junctional proteins (the inner blood retinal barrier) (see text for details) ILM= Internal Limiting Membrane, GCL=ganglion cell layer, INL= inner nuclear layer, PR and ONL= photoreceptors and outer nuclear layer, RPE= retinal pigment epithelium

Therapeutic intervention at the molecular level (i.e. anti-VEGF treatment) (Cunningham *et al.* 2005) would attenuate the principal cytokine in this molecular cocktail, but that effect may not be completely beneficial in resolving macular oedema because other angiogenic cytokines and proteolytic systems with the continued lack of anti-angiogenic influence may still permit the damage of the blood retinal barrier.

CHAPTER 4.4 CELL WORK AND PERMEABILITY STUDIES

Changes in the junctional staining of ZO-1 and β-catenin by VEGF were seen in the primary retinal endothelial cells which concurs with findings seen in brain microvessel endothelia for changes in ZO-1 (Wang *et al.* 2001) and in bovine pulmonary artery endothelial cells for the changes in β-catenin (Cohen *et al.* 1999). The changes seen in our primary cultures were at a VEGF concentration of 100 ng/ml, but at lower concentrations (found in the ocular fluids of the patients studied) only minimal or no obvious changes of ZO-1 and β-catenin junctional distribution was seen. VEGF-A is able to induce phosphorylation of ZO-1 and other tight junction proteins including occludin. Such biochemical change in these proteins induces redistribution of these proteins away from the junction (Antonetti *et al.* 1999) (Antonetti *et al.* 1998). Interestingly, HGF also produced similar minimal changes. Furthermore the vitreous of the patients studied (Group 1 with dome-shaped macular profile and Group 2 with a diffusely-elevated profile) demonstrated very minimal change in the distribution of these junctional proteins. This would suggest that the vitreous may contain the relevant cytokines and at an appropriate concentration capable of inducing junctional changes in the endothelial cells but in these primary endothelial cells VEGF at 100 ng/ml produced the greatest effect on ZO-1. However as there was insufficient vitreous from reference patients (macular hole) to test on the JG2 cells in a similar manner, we are unable to conclusively and objectively conclude that the diabetic vitreous contained the relevant cytokines at the appropriate concentration to induce the endothelial junctional changes.

The immortalized retinal endothelial cell line, JG2, also showed changes in the junctional staining of ZO-1 after VEGF-A and HGF at a gradation of concentrations, and vitreous from clinical Groups 1 and 2. These changes were seen both after 30 minutes and 24 hours of treatment with the relevant cytokine / vitreous. The changes seen with HGF may also suggest that this cytokine is also responsible for alteration of junctional molecules, promoting paracellular permeability. As these alterations in ZO-1 were also seen on addition of vitreous from a diabetic patient, it would suggest that there are vasoactive cytokines at a sufficient concentration to promote this alteration.

Given that both VEGF-A and HGF was found in the vitreous of the diabetic patients enrolled into the study, then both these cytokines in accordance with the cell culture results may prove important mediators of paracellular permeability. However other cytokines e.g. MMP 9 may also contribute to the breakdown of endothelial tight junctions.

Addition of VEGF seemed to increase changes in the actin fibres, producing stress fibres and condensation of actin fibres, particularly actin spanning the cell borders of the cell (cortical actin). Similar findings have been demonstrated previously (Rousseau *et al.* 1997). However on comparison of the treated cells with the non-treated cells, the actin staining seemed similar.

As mechanisms other than paracellular leakage have been proposed, we also studied the induction of transcellular pathways after the addition of the cytokines. Caveolae are 50-80 nm detergent-insoluble membrane microdomains. They consist of flask-like invaginations of the plasma membrane that contain cholesterol and integral membrane protein caveolin. There are three known caveolin proteins (caveolin 1 to 3). Caveolins are a family of 22,000 Mr proteins and caveolin-1 appears to be an essential component of caveolae (Li *et al.* 1996). VEGF stimulation of bovine retinal endothelial cells led to increased endothelial Nitric Oxide Synthase (eNOS) activity via stimulation of VEGF receptor 2 (VEGF R2). Double-label immunofluorescence and cell fractionation procedures demonstrated co-localization of VEGF R2 and eNOS with caveolin-1 in caveolae plasma membrane. Associated with these findings was an increase in the permeability of the cellular monolayer by this trans-cytotic pathway, suggesting that VEGF signalling occurs *via* the caveolar compartment (Feng *et al.* 1999). The staining of caveloin-1 after VEGF stimulation in the JG2 cells that demonstrated an increased perinuclear distribution of caveolin-1 after 30 minutes and 24 hours of VEGF-A treatment probably represents the activation of VEGF receptor 2 signalling *via* the caveolar compartment. Renal microvascular endothelial cells also showed an increase in the number and diameter of caveolae as assessed by transmission electron microscopy after 30 minutes of VEGF activation (Chen *et al.* 2002). Interestingly this response seemed to be biphasic, maximal at the highest and lowest VEGF-A concentrations studied. Further work especially in primary cultures would need to be conducted to confirm this initial result.

The cell work described here suggested that there are changes in the ZO-1 junctional staining and redistribution of caveolin-1 after VEGF-A, HGF and diabetic vitreous stimulation in JG2 and primary endothelial cells. However, compared to the primary culture of rat retinal endothelial cells, the junctional staining in the JG2 differed in strength and clarity with the most obvious changes in ZO-1 staining occurring at the highest VEGF-A concentration in the primary cultures. Similarly, the permeability studies on the JG2 cells suggested that, in the cell system used, functional effects of the VEGF-A could not be ascertained. Therefore more work on the methodology of the JG2 cultures and the optimal substrate and culture conditions are needed before this immortalized cell line can be used to study the structural and functional effects of diabetes-related cytokines at the cellular level.

Indeed from these results either from primary cultures or the JG2 cell line, it would suggest that the sensitivity of the cells to junctional alteration by cytokines is not particularly good as at only 100ng/ml of VEGF was there an appreciable (and slight) change in the distribution of ZO-1. This could be because the VEGF receptors maybe structurally altered or physically unavailable for VEGF to bind to in this model, or in relation to the addition of vitreous to the cell culture, the action of VEGF and other cytokines maybe diminished as they could be bound to vitreous proteins and unable to bind to their respective receptors as opposed to pure cytokine addition to the cell line, or finally as in the case of the JG2 cells which showed great morphological variablilty not only between cell plates and culture but also in the same cultute plate suggesting these cells are not of optimal and reliable quality in the present culture and media protocol to use to assess junctional distribution of proteins by cytokines.

The assessment of the junctional, actin and caveolae staining was based on a subjective evaluation of the staining. Therefore the interpretation of these staining results needs to be further confirmed by additional work with western blotting and / or quantitative fluorescence in future work. Furthermore this work confirmed that there are limitations of the JG2 cell line in order to assess junctional staining. Therefore primary cultures may provide a valuable substitute in evaluating the junctional response of the retinal endothelial cells to cytokines.

CHAPTER 4.5 PATHOGENESIS OF MACULAR OEDEMA

Experimental animal work *in vivo* using fluorescent microspheres in 1 week diabetic rats has suggested that VEGF initiated breakdown of the blood-retinal barrier in the diabetic retina involves changes in the venules and capillaries of the superficial inner retinal circulation (Qaum *et al.* 2001), but as the duration of diabetes increases, the changes become more extensive and involve arterioles of the inner circulation and extend throughout the post-capillary venules and into the capillary bed of the deeper (outer plexiform) retinal circulation using concanavalin-A staining (Barber and Antonetti 2003). Extrapolating these data to diabetic patients with macular oedema without corresponding human data, is fraught with problems, nevertheless the animal work does confirm that extensive changes occur in the retinal circulation in the diabetic state producing leaky vessels and macular oedema.

The increased permeability of these vessels may result from change at the endothelial cell level (within junctional proteins), which probably show increased paracellular permeability from changes in the tight junctions brought about the action of VEGF – A and HGF in the retinal microenvironment. There may also be increased transcellular permeability via the action of caveolae, which could also be acting as intracellular vehicles transporting VEGF receptor 2 to the endothelial nucleus to instigate transcriptional (Feng *et al.* 1999) and other intracellular changes. VEGF may be acting unopposed as changes in the antiangiogenic agents (soluble flt-1 receptor and PEDF) begin to occur in diabetes together with the changes in angiopoietin levels. As angiopoietin 1 levels decrease in relation to angiopoietin 2 concentrations, the vascular endothelium becomes hostage to the permeabilizing effects of VEGF augmented by angiopoietin 2, as both the stabilizing effects of angiopoietin 1 and the inhibitory effect of soluble flt-1 receptor and PEDF recede. Other growth factors also begin to take effect upon the retina - notably MMP 9 and TGF-β1. Both can work in concert to induce either an increase in permeability or together with changes already occurring in the biochemistry of the diabetic vitreous to change the vitreomacular anatomy, eventually leading to vitreomacular traction. Haemodynamic disturbance especially with the changes in endothelin-1 are also seen in NPDR. The decrease in endothelin-1 in NPDR as described here may allow for

vasodilatation and therefore an increased capillary hydrostatic pressure. This through its disturbance of Starling's equilibrium would allow for accumulation of extracellular fluid especially in the presence of a changing, disturbed and leaky endothelium.

However as these changes proceed at the vascular endothelial level, there may also be a disturbance of the neuroretina occurring in tandem or even preceding the vascular changes.

Possibly central to the changes in the neuroretina is the disturbance of the Müller cell. The mechanism may involve the effect of the ischaemia and hypoxia of diabetic retinopathy on the Müller cell. Such a metabolic alteration leads to increased VEGF-A and decreased PEDF production (Eichler *et al.* 2004) as well as changes in the distribution and number of potassium (K^+) channels in the Müller cell. There are two types of potassium channels, with one type strongly associated with a water channel called aquaporin 4 (Bringmann *et al.* 2004). There is a K^+ channel which faces retinal neurons and facilitates removal of excess neuronal-derived potassium from the extracellular space. This results in a change in the membrane potential of the cell allowing for an instantaneous opening of K^+ 4.1 subtype channels which are mainly located on the Müller cell membrane surrounding the retinal vessels and internal limiting membrane. Associated with this 4.1 subtype is the water channel aquaporin 4 (Aq 4). The outward movement of potassium into the retinal capillaries and vitreous via the 4.1 channel is accompanied by a flux of water out of the cell through the Aq 4 channel into the capillaries and vitreous. Diabetic retinal ischaemia and hypoxia can produce down-regulation of the potassium 4.1 subtype. This would lead to a decrease in the outward movement of potassium whilst the inward movement from the neurons would continue resulting in reversed osmotic gradient driving water through the aquaporin 4 channels into the cell, producing intracellular oedema. This in turn impairs transglial potassium clearance from the extracellular space resulting in a subsequent change in the osmotic drive allowing for the development of extracellular oedema (Fig 4.1). The combination of extracellular ad intracellular oedema defines the clinical picture of macular oedema. This degree of retinal ischaemia and hypoxia of NPDR also increases VEGF-A production and decreases both the PEDF and sFlt-1 production (Fig 4.2). The Müller cell is central to this change in balance of the angiogenic and antiangiogenic profile (Eichler *et al.* 2004). As the sFlt1 decreases in

NPDR its attenuating effect on VEGF-A is reduced allowing for VEGF-A to predominate especially on the retinal capillaries increasing permeability and disturbing extracellular homeostasis and the Müller cell's ability to counteract the increased water flux as its potassium channels have been disturbed and overwhelmed resulting in the development of macular oedema, both of the extracellular and intracellular type. Once the PEDF concentration falls below a critical safety level then the full angiogenic drive of VEGF is unleashed producing active PDR (Fig 4.2)

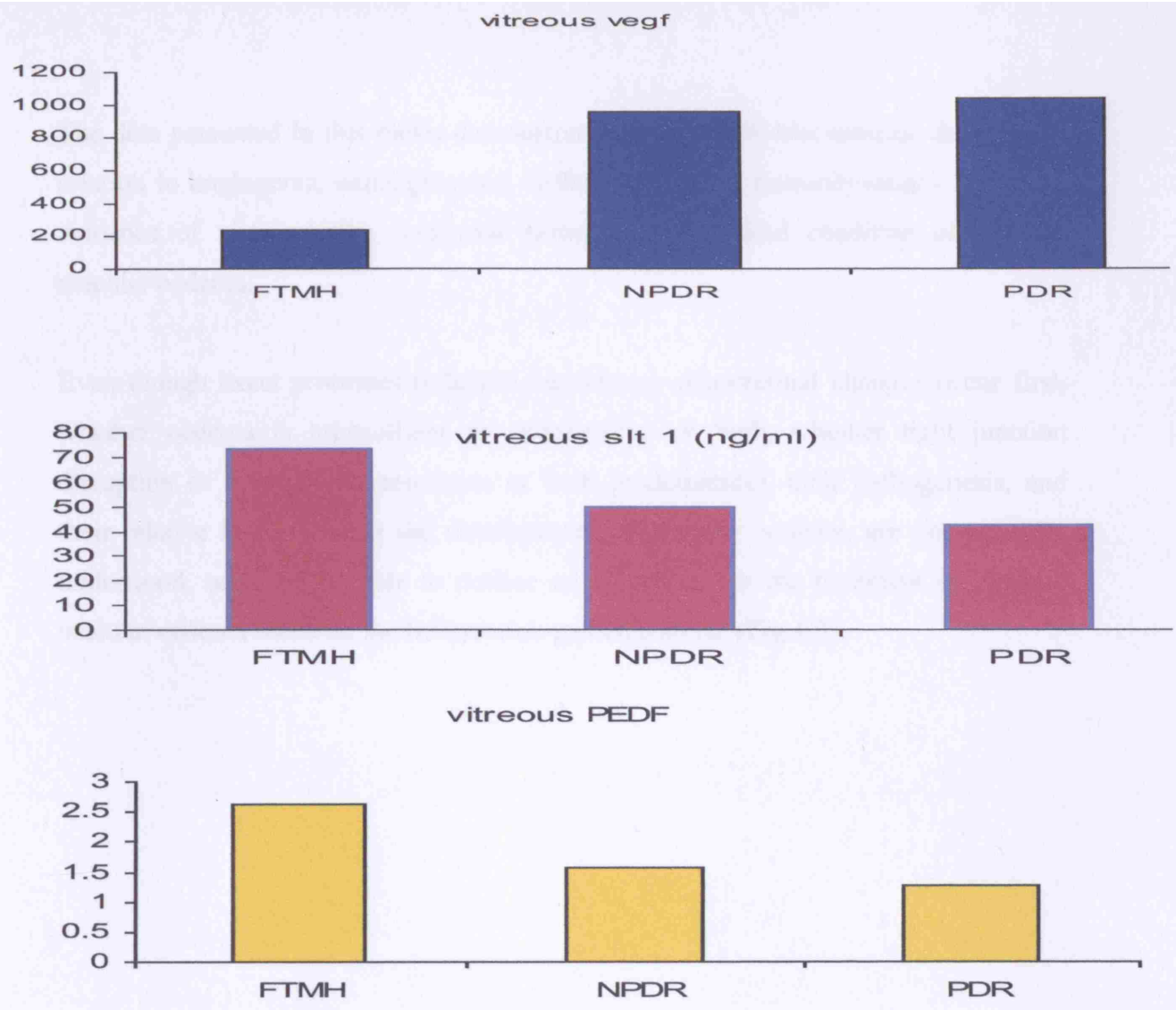


Fig 4.2

Fig 4.2. The changing balance of VEGF-A and anti-angiogenic cytokines in Diabetic Retinopathy.

The data presented in this thesis demonstrates the cytokine-biochemical changes (in relation to angiogenic, antiangiogenic, inflammatory and haemodynamic) and OCT evidence of vitreomacular tractional forces in the clinical condition of diabetic macular oedema.

Even though exact processes (whether vascular or neuroretinal changes occur first, whether oedema is intracellular or extracellular or both, whether tight junction disruption or transcellular processes or both predominate), their pathogenesis, and their relative importance in the development of macular oedema, are not yet fully understood, one may be able to outline an algorithm for the treatment of diabetic macular oedema based on the information gathered so far (**Fig 4.3**).

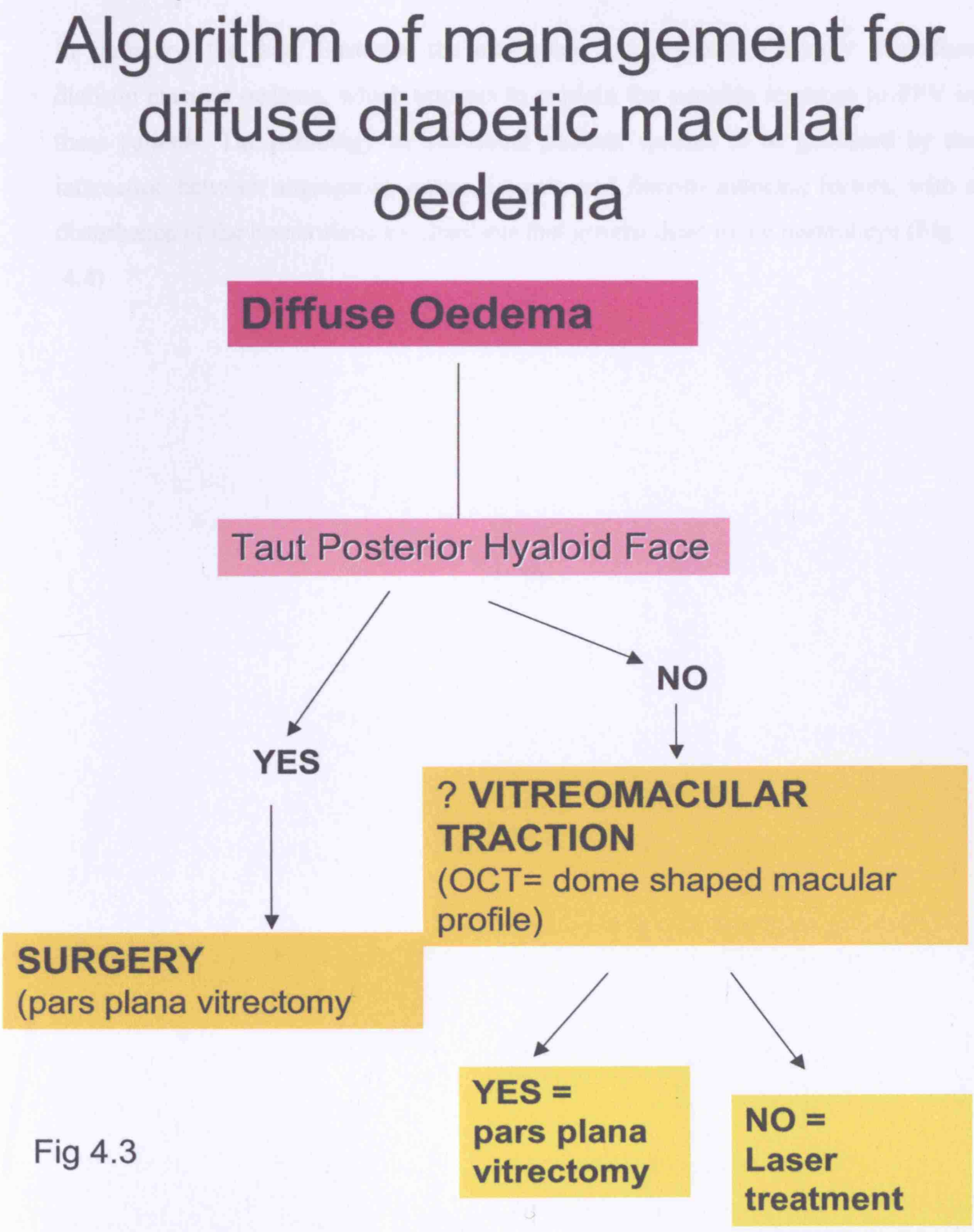


Fig 4.3. Potential management algorithm for diabetic macular oedema

CHAPTER 4.6 SUMMARY

In summary, the data illustrates the molecular and structural diversity in diffuse diabetic macular oedema, which appears to explain the variable response to PPV in these patients. The pathology in individual patients appears to be governed by the interaction between angiogenic, antiangiogenic and fibrosis-inducing factors, with a disturbance of the homeostatic mechanisms that govern them in the normal eye (**Fig 4.4**)

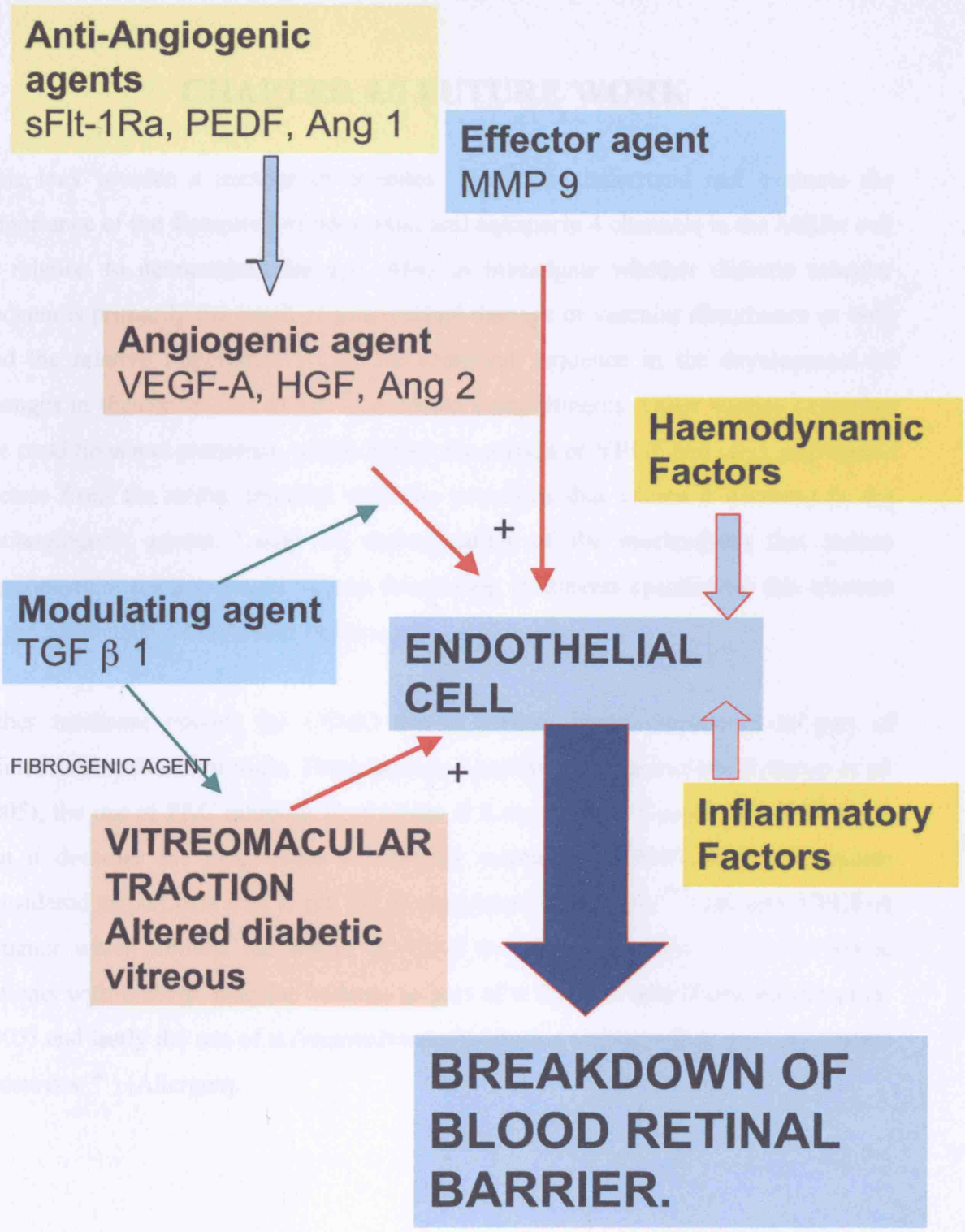


Fig 4.4

Fig 4.4. Potential pathways leading to blood retinal-barrier breakdown. The large number of influences direct or indirect on the endothelial cell which induces breakdown of the blood retinal barrier.

CHAPTER 4.7 FUTURE WORK

This may involve a number of avenues. Firstly to understand and evaluate the importance of the disruption of potassium and aquaporin 4 channels in the Müller cell in relation to neuroretinal damage. Also to investigate whether diabetic macular oedema is primarily the result of neuroretinal damage or vascular disturbance or both and the relative importance if not the temporal sequence in the development of changes in these two distinct yet interrelated compartments. Other studies exploring the conditions and processes, which induce the release of VEGF and other angiogenic factors from the retina, together with the processes that causes a decrease in the antiangiogenic agents. Lastly an understanding of the mechanisms that induce vitreomacular traction would help in developing treatments specific for this element in the pathogenesis of macular oedema.

Other treatment options for CSMO are at present being considered as part of randomised multicentre trials. These include intravitreal trimacinolone (Larsson *et al.* 2005), the use of PKC inhibitor to examine if it can prevent visual loss in CSMO or can it decrease the progression of macular oedema to CSMO. Other treatments considered as part of a trial is the use of pegaptanib (Macugen ™) (an anti-VEGF-A aptamer which inhibits the action of VEGF-A, particularly the 165 isoform)) in patients with diabetic macular oedema as part of a Phase 2 trial (Cunningham *et al.* 2005) and lastly the use of a dexamethasone posterior segment drug delivery system (Posurdex ™) (Allergen).

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Patel, JI., Hykin, PG., Gregor, ZJ., Boulton, M., Cree, IA. (2005). Angiopoietin concentrations in diabetic retinopathy. Br J Ophthalmol **89**: 480-483.

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Vitreous and aqueous concentrations of proangiogenic, antiangiogenic factors and other cytokines in diabetic retinopathy patients with macular edema: Implications for structural differences in macular profiles

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